Regulatory Functions of Sialylated Glycans and Gut Microbiota in Mucosal Immunity

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von

Yen-Lin Eddie Huang

aus

Taiwan

Promotionskomitee

Prof. Dr. Thierry Hennet (Vorsitz)

Prof. Dr. Martin Hersberger

PD. Dr. Lubor Borsig

Dr. Christophe Chassard

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SUMMARY

The intestinal microbiota regulates physiological processes and maintains homeostasis in the gut. Accordingly, research is conducted to understand the factors influencing the composition of the intestinal microbiota. Dietary- and host-derived glycans have been shown to profoundly affect the growth of the intestinal microbiota. However, the specific link between glycans and intestinal microbiota remains unclear. The focus of this work was to determine how glycans regulate the intestinal microbiota and thereby influence the host mucosal immunity.

Sialic acids, provided by milk oligosaccharides and mucosal glycans, are preferred nutrient sources of *Escherichia coli* in the intestine. We identified increased sialidase production by the commensal *Bacteroides vulgatus* during intestinal inflammation. This led to increased free sialic acid in the colon during inflammation thereby promoting the proliferation of *E. coli*. The proliferation of *E. coli* exacerbated inflammatory responses by stimulating the production of pro-inflammatory cytokines by intestinal dendritic cells. By inhibiting intestinal sialidases, the proliferation of *E. coli* was prevented and intestinal inflammation is attenuated. This finding demonstrates that sialic acid catabolism influences the intestinal microbiota and mucosal immunity.

Furthermore, we investigated the links between the gut microbiota, their metabolites and mucosal immunity. The major bacterial fermentation products, namely short-chain fatty acids, have been measured and compared between healthy and colitogenic mice. A significant reduction of caecal short-chain fatty acids, especially butyrate, was observed during intestinal inflammation. This correlated with lower abundance of butyrate-producing bacteria such as *Faecalibacterium prausnitzii* and *Eubacterium hallii.* Additionally, by treating mice with antibiotics, intestinal inflammation was exacerbated whereas caecal short-chain fatty acid levels decreased. By exogenously supplying colitogenic mice with butyrate, inflammation was attenuated, therefore confirming the beneficial role of butyrate in regulating mucosal immunity.

Our studies demonstrate for the first time that host-derived glycosylation confers a critical growth advantage for *E. coli* during intestinal inflammation. Furthermore, by establishing the essential contribution of intestinal sialic acid and sialidase in mediating microbial dysbiosis, we define a fundamental mechanism by which *E. coli* proliferates in the gut, thereby unraveling a novel therapeutic target for the treatment of intestinal inflammation. Overall, our studies reveal potential opportunities for the treatment of inflammatory intestinal diseases by regulating glycan metabolic pathways and the gut microbiota.

ZUSAMMENFASSUNG

Die intestinale Mikroflora reguliert einerseits physiologische Prozesse und andererseits sorgt es für Homöostase im Darm. Aufgrund dessen wird nach Faktoren geforscht, welche die Zusammensetzung der Mikroflora im Darm beeinflussen. Glykane sind eine wichtige Nahrungsquelle und beeinflussen das Wachstum der intestinalen Mikrobiota. Die exakten Zusammenhänge zwischen bestimmten Glykanen und den einzelnen Komponenten der Mikrobiota sind jedoch weiterhin unklar. In dieser Arbeit wird gezeigt wie Glykane die mikrobielle Zusammensetzung im Darm verändern und damit auch die Immunabwehr an den Schleimhäuten des Darms beeinflussen.

Sialinsäuren stellen eine gute Nahrungsquelle für *Escherichia coli* dar. Im Darm stammen Sialinsäuren zum einten aus der Ernährung (z.B. Milch Oligosaccharide des Menschen) und zum anderen kommen sie in Schleimhaut produzierten Glykanen vor. Wir konnten feststellen, dass die Sialidase von *Bacteroides vulgatus*, während inflammatorischen Darmerkrankungen, in erhöhten Mengen vorhanden ist. Dies führte zu einem erhöhten Anteil an freien Sialinsäuren, welches wiederum die Vermehrung von *E. coli* förderte. Die erhöhte Proliferation von *E. coli* führte wiederum zu einem verstärkten inflammatorischen Zustand, aufgrund einer erhöhten Produktion an pro-inflammatorischen Zytokinen durch intestinale dendritische Zellen. Durch die Inhibition von intestinalen Sialidasen, wurde die Proliferation von *E. coli* verhindert und die Entzündung abgeschwächt. Diese Studie zeigt damit, dass der Katabolismus von Sialinsäuren die intestinale Mikrobiota und damit auch das Immunsystem an der Darmschleimhaut beeinflusst.

Weiterhin wurde der Zusammenhang zwischen der Mikrobiota im Darm, ihrer Metaboliten und dem mukosalen Immunsystem untersucht. Die kurzkettigen Fettsäuren bilden den Hauptteil der bakteriellen Fermentationsprodukte und wurden daher in ihrer Menge zwischen gesunden und kolitischen Mäusen verglichen. In Kolitis erkrankte Mäuse war die Menge an kurzkettigen Fettsäuren, insbesondere Butyrat, im Dickdarm signifikant reduziert. Dies korrelierte mit geringeren Mengen an den Butyrat-produzierenden Bakterien *Faecalibacterium prausnitzii* und *Eubacterium hallii*, die wir beobachtet haben. Zusätzlich

konnte durch die Behandlung von Mäusen mit Antibiotika die Kolitis-Erkrankung verstärkt werden wobei die Menge an kurzkettigen Fettsäuren abnahm. Durch die exogene Versorgung von kolitogenen Mäusen mit Butyrat, wurde die Entzündung verringert, wodurch die vorteilhafte Wirkung von Butyrat, auf die Regulation des Immunsystems der Schleimhaut, bestätigt werden konnte.

Unsere Studien demonstrieren erstmalig, dass die Glykosylierung des Wirts, dem Bakterium E. coli einen kritischen Wachstumsvorteil ermöglicht. Weiterhin, zeigen wir, durch die Ermittlung der essentielen Rolle von intestinaler Sialinsäure und Sialidasen während mikrobieller Dysbiose, einen fundamentalen Mechanismus für die Proliferation von E. coli im Darm. Hiermit haben wir einen neue Möglichkeit aufgedeckt für die Behandlung von Kolitiserkrankungen. Insgesamt weist unsere Studie auf neue Möglichkeiten für die Behandlung von inflammatorischen Kolitiserkrankungen durch die Regulierung von Glykan-metabolischen Reaktionen und der intestinalen Mikrobiota.

ABBREVIATIONS

3SL	α2,3-sialyllactose
4MuNeuNAc	2’-(4methylumbelliferyl)-α-D-N-acetylneuraminic acid
6SL	α2,6-sialyllactose
CD	Crohn’s disease
CF	Cecal fluid
C.F.U.	Colony forming unit
DC	Dendritic cell
DGGE	Denatured gradient gel electrophoresis
DSS	Dextran sulfate sodium
Fuc	Fucose
Gal	Galactose
GalNAc	N-acetyl galactosamine
GALT	Gut associated lymphoid tissue
GI	Gastrointestinal tract
Glc	Glucose
GlcNAc	N-acetylglucosamine
HMO	Human milk oligosaccharides
HPAEC	High performance anion exchange chromatography
IBD	Inflammatory bowel disease
INF-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IRAK	Interleukin 1 receptor-associated kinase
IRF3	Interferon-regulatory factor 3
Ικκ	IκB kinase
LPS	Lipopolysaccharide
M cell	Microfold cells
MHC	Major histocompatibility complex

MLN	Mesenteric lymph node
MyD88	Myeloid differentiation primary response gene 88
NanT	Neuraminate transporter
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Nucleotide-binding domain and leucine rich repeat containing receptor
NOD	Nucleotide-binding oligomerization domain
PRR	Pattern recognition receptor
sIgA	Secretory immunoglobulin A
Sia	Sialic acid
ST	*St3gal4* glycosytransferase
Stp	Streptomycin
T_{reg}	T regulatory cell
TGF-β	Transforming growth factor beta
T_H1	T helper 1
T_H2	T helper 2
TLR	Toll like receptor
TNF-α	Tumor necrosis factor alfa
UC	Ulcerative colitis
Van	Vancomycin
WT	Wildtype
XF	Cross-fostering

INTRODUCTION

1. Nutrition and immune system

Human milk is widely considered as a gold standard for the infant's early nutrition. It provides all the components that are necessary for proper growth and development of neonates. Human milk contains diverse macronutrients including carbohydrates, proteins, lipids and other micronutrients such as vitamins and minerals. Besides nutritional benefits, human milk also contains multiple bioactive and immunomodulatory components. These bioactive components provide active and passive protection for neonates. Beneficial effects of breast-feeding are well documented in several studies, including reduced risks of infectious diseases, necrotizing enterocolitis (NEC) [1], food allergies and atopic diseases [2]. Moreover, breast-feeding also provides a long-lasting effect, as shown by a lower incidence of developing type II diabetes [3], metabolic syndrome diseases [4], and inflammatory bowel diseases (IBD) [5].

1.1 Nutritional components in milk

The composition of human milk, unlike standardized formula, is dynamic and differs over the lactation period and between individuals. The main macronutrients of milk are carbohydrates, lipids, and proteins. The carbohydrate fractions in both formula and breast milk are mainly composed of the disaccharide lactose. However, human milk is also a rich source of unique milk oligosaccharides (HMOs) which are thus lacking in manufactured formula. The unique compositions and biological functions of HMOs will be discussed later (**Ch 1.3-1.4**).

The protein fractions of human milk are mainly divided into soluble whey protein and insoluble casein [6]. Whey protein is the collection of globular proteins that mainly consists of β-lactoglobulin, α-lactalbumin, serum albumin, and immunoglobulin. Soluble whey proteins are readily digestible for infants and are also abundant sources of essential amino acids [6-7]. Casein is the collection of phosphoproteins, which constitute about 80% of total

milk proteins in bovine milk, and mainly act as a mineral carrier for calcium and phosphorus in promoting their digestibility in the stomach. Casein-derived peptides have been shown to possess antimicrobial [8], antioxidant, [9] and antihypertensive activities [10].

The lipid fractions of milk are the major energy sources for neonates, accounting for about 50-60% of the total calories intake [11]. It consists of mainly triacylglycerol (~97%), diacylglycerol (2%), free fatty acids (~1%), and cholesterol (<0.5%) [12]. The composition of lipids in milk is highly dependent on maternal diet, therefore, varies between individuals and over lactation period. Recently, fatty acids have received more attention because of their health benefits to the development of neonatal nervous system and immune system. The most abundant fatty acids in human milk are oleic acid (C18:1) and palmitic acid (C16:0) [13]. Moreover, human milk is also rich in polyunsaturated fatty acids (PUFAs) including omega-3 and omega-6 fatty acids. They are essential fatty acids that can only be acquired from the diet, but cannot be biosynthesized *de novo*. The importance of PUFAs is shown by the essential roles of docosahexaenoic acid (DHA) and arachidonic acid (AA) in proper development of brain and nervous system [14], and the immunomodulatory effect of DHA in treatment of inflammatory bowel disease [15].

1.2 Bioactive components in milk

Human milk is rich in bioactive components, which include various growth factors and immunological factors. These bioactive components contribute to active and passive protection for neonatal immune system which is not yet fully developed. Moreover, immunological factors are responsible for modulating the immune tolerance and maintaining homeostasis of postnatal innate and adaptive immunity. The multiple functions of bioactive components in human milk are summarized in **Table 1**. Human milk contains various hormones and growth factors that support growth and development in systemic level. Epidermal growth factor (EGF), which is enriched in colostrums, plays multiple roles in promoting maturation of the intestinal mucosa. EGF promotes the proliferation of enterocytes, facilitates mucosal repair, and also provides protection against NEC [16]. Insulin-like growth factor also promotes intestinal epithelial growth and regulates glucose

metabolism [17]. Neuronal growth factor supports neuronal outgrowth and survival. Growth-supporting hormones, such as erythropoietin (EPO), reaches receptors in the intestinal tract and stimulate the erythrocytes production in bone marrow [18]. Other hormones like calcitonin and adiponectin regulate calcium metabolism and energy conversion, respectively.

Table 1. Bioactive components and their functions in human milk (adapted from [19-20]).

Bioactive components and functions in milk

Growth & development	Antimicrobial activity	Immunomodulation
• Growth factors EGF, VEGF, NGF, IGF Erythroprotein	• Immunoglobulins (soluble IgA, IgG, IgM)	• Immune cells (Macrophage, Neutrophil, Lymphocyte)
• Hormones Calcitonin, somatostatin Adiponectin, Leptin	• Antimicrobial proteins Lactoferrin α-Lactalbumin, κ-Casein Defensin Complement Lysozyme	• Cytokines - pro-inflammatory (IL-1β, IL-6, IL-8, TNFα, IFNγ) - anti-inflammatory (IL-10, TGFβ)
• Cholesterol and Fatty acid (AA, DHA, LCPUFA)	• Soluble decoy receptors sTLR, CD14	• Chemokines MIF, CCL5, MCP-1, MCP-3
• Ganglioside, HMOs	• HMOs and Mucins	• Receptors sTLR, IL-1R, IL-6R, TNF-R
• Vitamins		

EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; NGF, nerve growth factor; IGF, insulin-like growth factor; AA, arachidonic acid; DHA, docosahexaenic acid; LCPUFA, long-chain polyunsaturated fatty acids; HMOs, human milk oligosaccharides; MIF, macrophage migration inhibitory factor; CCL5, C-C Chemokine ligand 5; MCP-1, monocyte chemotactic protein-1

Human milk contains a wide range of immunological factors including immune cells, immunoglobulins, antimicrobial proteins and cytokines which mutually facilitate the development of infant's immunity. Immune cells including macrophages (55-60%), neutrophils (30-40%), and lymphocytes (5-10%) remain viable in breast milk [21]. Macrophages and neutrophils provide a direct pathogen clearance activity and activate the immune system. In addition, milk-derived lymphocytes have been shown to traverse the

neonatal intestine and promote T cell development as shown by an increase size of thymus in breast-fed infants [22]. At birth, immunoglobulin levels are very low in the intestinal mucosa of neonate [23]. Therefore, the early response to dietary and microbial antigens in neonatal mucosal immune system relies on maternal milk. Secretory immunoglobulin A (sIgA), which constitutes 90% of total immunoglobulins in milk, inhibits the adherence of enteric pathogens, neutralizes toxins, and induces the immune tolerance. Other components of innate immunity exhibit direct bactericidal activity such as lactoferrin, lysozyme, α-lactalbumin, and defensins [24]. Soluble decoy receptors like sTLR2 and sCD14 [25-26] also prevent pathogen adherence and infection [27].

Cytokines and chemokines also show immunomodulating activities. The primary source of milk cytokines is the mammary gland, whereas small amounts of cytokines are secreted by leukocytes [24]. A variety of cytokines and chemokines in human milk regulate the induction of pro- or anti- inflammatory responses [28]. Pro-inflammatory cytokines like IL-1β, IL-6, IL-8, and TNF-α mediate inflammatory process and clearance of pathogens [29]. Anti-inflammatory cytokines, such as IL-10 and TGF-β, dampen the excessive inflammatory responses and promote healing of the intestinal mucosa [30-31]. Corresponding soluble receptors and cytokine antagonists (e.g. IL-1R, IL-6R, TNFR) also contribute to anti-inflammatory properties [32]. Chemokines like CCL2-5 and CXCL1-3 families are also identified in milk and facilitate the recruitment of leukocytes to the site of infection [28]. Overall, bioactive components and immunological factors not only provide direct health benefits but also instruct the development of postnatal immune system.

1.3 Composition of milk oligosaccharides

The composition of human milk is unique among mammals because of the high abundance and complexity of human milk oligosaccharides (HMOs) [33]. HMOs are composed of five main monosaccharides, including glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), *N*-acetylneuraminic acid (Neu5Ac) and fucose (Fuc). The biosynthesis of HMOs depends on the expression of glycosyltransferases in mammary gland, and typically begins with the formation of a lactose core. Lactose or the elongated oligosaccharides can be

further fucosylated in α1-2, α1-3, α1-4 linkages and sialylated in α2-3, α2-6 linkages at the terminal positions. The proportion of fucosylated, sialylated, and neutral HMOs in human milk ranging from 35-50%, 12-14%, and 42-55%, respectively and varies between individuals [34]. The structural diversity of HMOs are summarized in **Figure 1**.

The majority of HMOs are not digestible and absorbable in the human intestine due to lack of suitable glycosidase enzymes in the GI tract. Therefore, a major question is why does the mammary gland synthesize energetically costly HMOs that do not directly provide a nutritional benefit to the infants? Originally, HMOs were defined as prebiotic factors that selectively enrich a group of bifidobacteria, which confer beneficial advantages to the breast-fed infants. Nowadays, accumulating evidences suggest that HMOs not only function as a metabolic source for desired bacteria but are also involved in regulating gut homeostasis and mucosal immunity. The postulated beneficial effects of HMOs can be divided into the following categories: (1) HMOs serve as prebiotics promoting the colonization of beneficial bacteria; (2) HMOs serve as soluble decoy receptors preventing the attachment and infection of pathogens; (3) HMOs modulate intestinal epithelial cell functions; (4) HMOs are critical nutrients for brain development.

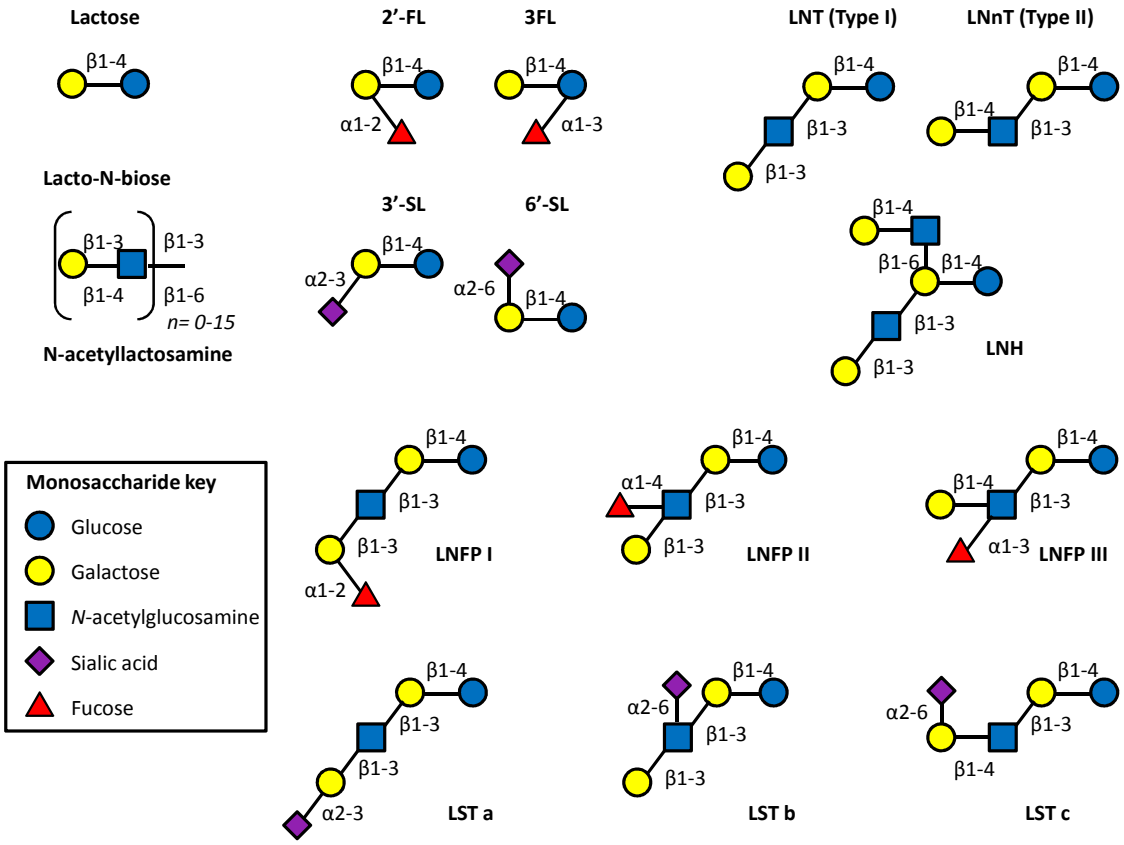


Figure 1. Structural diversity of human milk oligosaccharides (HMOs) (Adapted from [35]). HMOs consist of a lactose core linked to lacto-N-biose or to *N*-acetyllactosamine with repeating units. Lactose can be sialylated or fucosylated by different linkages to form trisaccharides 3SL, 6SL, 2FL, and 3FL. Lactose can also be elongated in repeats of lacto-N-tetrose (LNT) or *N*-acetyllactosamine (LNnT). Elongated type I or II chains can be fucosylated in different linkages to form a variety of structural isomers. The elongated chains can be sialylated in different linkages to form structural isomers.

1.4 Functions of milk oligosaccharides

1.4.1 HMOs function as prebiotics

Prebiotics are ingredients, which provide as selective nutrient sources for a desired group of commensal bacteria that contribute to the health of a host. HMOs are resistant to acidic and enzymatic hydrolysis in the stomach and small intestine, thereby the majority of HMOs can reach the colon that harbors the densest microbial community in the GI tract. To utilize HMOs, bacteria require an array of glycosidases, For example, α-fucosidase or α-sialidase are responsible for the initial step for cleaving terminal fucose or sialic acid on HMOs. Afterward,

the remaining structures are available for digestion by β-hexosaminidase or lacto-N-biosidase to generate monosaccharides and disaccharides. Intestinal bacteria which possess the required glycosidases thus receive a growth advantage on colonization of the host colon.

It has been shown that bifidobacteria are predominant species in the gut of breast-fed infants compared to formula-fed infants [36-37]. *Bifidobacterium longum subsp. Infantis* (*B. infantis*) and *B. breve* have been shown to grow on HMOs as a single carbon source in vitro [38-39], and preferentially utilize fucosylated and sialylated HMOs. Additionally, *B. bifidum* grows slower on HMOs compared to *B. infantis* [40] while *B. longum subsp.* only grow on lacto-N-teteraose [41]. The different growth profiles among bifidobacteria suggest that they metabolize HMOs through different pathways. It has been described that *B. infantis* and *B. breve* encode glycans transporters and intracellular glycosidases, indicating that HMOs are internalized and metabolized in cells [42-43]. By contrast, *B. bifidum* expresses extracellular glycosidases that cleave HMOs and then transport the disaccharide into cell. In addition to bifidobacteria, recent studies have shown that some *Lactobacillus sp.* are able to utilize fucosylated HMOs [44], and several *Bacteroides* spp. also demonstrate a robust growth on HMOs [45-46]. The dominance of bifidobacteria in breast-fed infants limits the nutrient supply for other bacteria, thereby preventing the growth of potentially pathogens in the newborns. Furthermore, bifidobacteria can protect from enteropathogenic infection through the production of acetate [47] and promote vitamins synthesis in mediating a trophic effect on the host intestinal epithelium [48]. Additionally, bifidobacteria stimulate mucosal immunity by enhancing IgA production against pathogens, like rotavirus [49]. Potential effects of prebiotics are summarized in **Figure 2**.

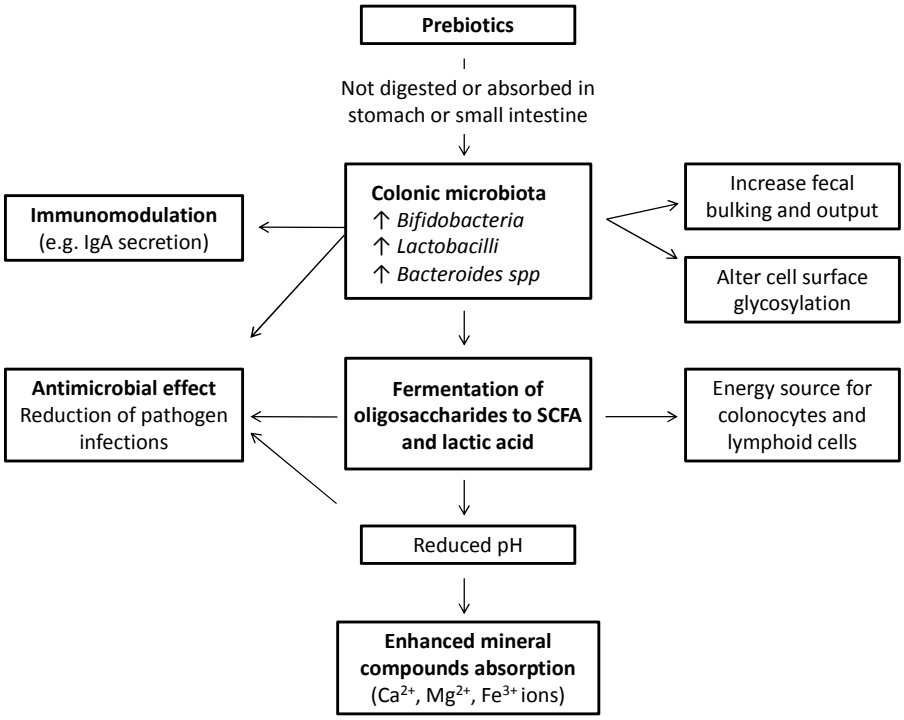


Figure 2. Effects of prebiotics in gastrointestinal tract (Adapted from [50]). Prebiotics promote the colonization of the gut by beneficial bacteria. These bacteria modulate gut homeostasis through multiple mechanisms, including the prevention of pathogens adherence, the production of beneficial nutrients (SCFAs, minerals and vitamins), and immunomodulation.

1.4.2 HMOs prevent pathogens and toxins adhesion

Adhesion of pathogens to the epithelial surface is an initial step of microbial infection. This binding is mediated through microbial lectin-glycan interaction [51]. Many HMOs share structural similarities to cell surface glycans because of common expression patterns of the glycosyltransferases [52]. Therefore, a variety of HMOs can serve as soluble decoy receptors, which reduce the adherence of pathogens on intestinal epithelial surface and prevent the ensuing infection. For instance, a recent study has shown that supplementation of exogenous fucosylated oligosaccharides reduces the binding of *Campylobacter jejuni* to intestinal epithelial cells *in vitro*, thereby inhibiting their colonization of the gut in mice [53]. Additionally, a human clinical study based on 93 mother-infant pairs suggested that infants exposure to higher concentration of 2FL correlated with a lower incidence of *C. jejuni*-associated diarrhea, and the protection against calicivirus diarrhea was associated with lacto-*N*-difucohexaose I (LDFH-I) [54]. This study has demonstrated that specific HMOs can

protect infants from specific pathogens induced diarrhea. However, studies also suggested that the protective effect of fucosylated oligosaccharides differs among individuals due to polymorphisms in the responsible fucosyltransferase genes [55-56].

In addition to fucosylated oligosaccharides, sialylated oligosaccharides are also abundant in human milk and play pivotal roles in neonatal protection. For instance, 3SL has been shown to inhibit the binding of bacteria or bacterial toxins, such as *Helicobacter pylori*, cholera toxin and *E. coli* enterotoxin to mucosa surface [57-58]. Disialyllacto-N-tetraose (DSLNT) has been also shown to prevent neonatal rats from NEC [59-60], and low level of DSLNT in maternal milk are implicated in the high incidence of developing NEC in the newborns. These anti-adhesive effects are not limited to bacterial infections but also observed in preventing virus infections, such as HIV [61-62], rotavirus [63], and norovirus [64]. Additional HMOs and other glycan structures inhibit the binding of pathogens are summarized in **Table 2**.

On the basis of these in vitro and in vivo studies, glycan structures which block specific pathogen adhesion are potential therapeutics against bacterial or virus infection. As a result, supplementation of beneficial oligosaccharides in infant's formula has recently received considerable interest to prevent the pathogenic infection in neonates. Advanced in large-scale synthesis of oligosaccharides has greatly facilitated the progression of clinical research.

Table 2. Pathogen recognition of host glycan receptors (Adapted from [58]).

Glycan receptor	Microorganisms	Bacterial toxins
α2,3 sialyllactose	S-fimbriated *E. coli*	Botulinum toxin B
	Helicobacter pylori	Cholera toxin AB5
	Rotavirus	Staphylococcal enterotoxin
Sia (α2,3) derivatives	Influenza virus A and B	
	Polyomavirus	
Sia (α2,6) derivatives	Influenza virus A and B	Pertussis toxin
Sia (α2,8) derivatives		Tetanus toxin
Sialylated oligosaccharides	*Streptococcus suis*	
	Enterotoxigenic *E. coli* (ETEC)	
	Uropathogenic *E. coli* (UPEC)	
Fucosylated oligosaccharides	*Campylobacter jejuni*	*E. coli* enterotoxin
	Clostridium difficile	
	Vibrio cholerae	
Glycosphingolipid	S-fimbriated *E. coli*	Cholera toxin AB5
GM1, GM2, GM3 (α2,3Sia)		Heat-labile Enterotoxin
GM1, GD1, GT1b		Botulinum toxin

1.4.3 HMOs modulate intestinal epithelial cell responses

Milk oligosaccharides directly modulate host intestinal epithelial cell functions by regulating cell surface glycosylation. Exposure of human colorectal epithelial Caco-2 cells with 3SL reduced the expression of sialyltransferases, therefore decreased α2,3- and α2,6-linked sialylation on the cell surface [65]. As a result, the changes of surface glycans reduced adhesion of enteropathogenic *E. coli* by 50% in vitro. In addition, milk oligosaccharides have also been shown to influence cell differentiation, proliferation and apoptosis [66]. Stimulation of intestinal epithelial cells (e.g. HIEC, HT-29, and Caco-2) with neutral or acidic milk oligosaccharides yielded a dose-dependent cell cycle arrest by inducing epidermal growth factor receptor signaling and cyclin B expression [67]. The results suggested that HMOs inhibit cell proliferation and cell cycle dynamic through mitogen-activated protein kinase (MAPK) signaling. However, whether this growth inhibition effect on intestinal cells correlates with the beneficial outcomes of breast-feeding requires further investigation. A

more recent study also demonstrated that 2FL and 6SL reduce cell maturation in an epithelial model of crypt-villus axis, suggesting a specific role of HMOs in mediating cell differentiation and maturation in the gut [68]. Overall, these observations strongly suggest that HMOs interact with epithelial cells, affect host gene expression and modulate cell differentiation and proliferation. However, whether such *in vitro* studies can translate to benefits for breast-fed infants remain to be investigated.

1.4.4 HMOs support development of the nervous system

Accumulating evidences suggest that sialic acid is an essential nutrient for brain development and cognition during pre- and post-natal stages [69]. Newborns have a lower capacity for the *de novo* synthesis of sialic acid that does not meet the high demand for rapid neural development [69]. Dietary sialic acid, may therefore facilitates neural development. Human milk is a rich source of sialylated glycans and sialic acid. Sialic acid is absorbed across the mucosa of the small intestine, and transported into the bloodstream [70-71]. Furthermore, sialic acid can be taken and utilized by a variety of human cells including epithelial, skin fibroblasts and neural cells [72] for synthesis of sialoconjugates. Additionally, a recent study demonstrated that gangliosides and sialylated glycoproteins are significantly higher in the brains of breast-fed infants compared to formula-fed infants, indicating the dependence of sialic acid on brain development [73]. Gangliosides and neural cell adhesion molecule (NCAM) are the most investigated sialoconjugates involved in neonatal brain development. Numerous studies support their involvements in mediating synaptic remodeling, neuronal plasticity, neural circuit formation and adhesive interaction during brain development [69, 74]. Nevertheless, whether sialylated HMOs are the primary sialic acid source that uphold brain development in breast-fed infants remains to be elucidated.

2. The gastrointestinal tract and mucosal immune system

2.1 Components of mucosal immune system

Human gastrointestinal tract is covered with a mucosal layer which directly and constantly exposed to dietary antigens and gut microbiota. Therefore, mucosal immune system requires appropriate mechanisms to distinguish commensal bacteria or food antigens from the pathogenic microorganisms. This complex interplay between the mucosal immune system and microbiota is mediated by intestinal epithelial cells (IECs) and gut-associated lymphoid tissues (GALTs) **(Figure 3)**.

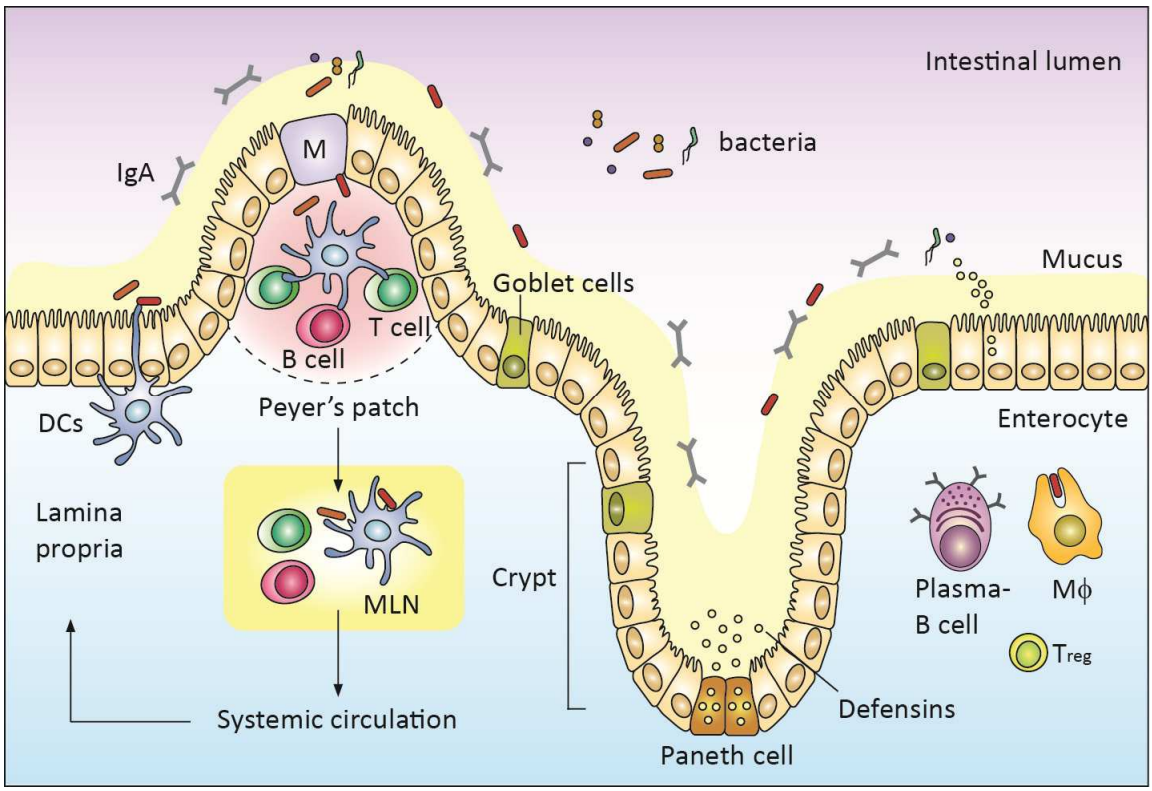


Figure 3. Intestinal epithelium barrier and GALTs (Adapted from [75-76]). The intestinal epithelial layer contains enterocytes, mucus-secreting goblet cells, antimicrobial proteins-releasing Paneth cells, and antigen delivering-microfold cells (M cells). M cells deliver antigens from the lumen to the subepithelial area in the Peyer's Patch where dendritic cells (DCs) recognize the antigens and present to T cells. Activated DCs and lymphocytes migrate via afferent lymph to the mesenteric lymph node (MLN). In MLNs, lymphocytes mature and enter systemic circulation and back to lamina propria (LP). LP macrophages kill bacteria directly through phagocytosis, and LP plasma B cells secrete IgA triggering antigen-specific immune response.

IECs serve as a physical barrier, separating luminal antigens from the underlying immune tissues and blocking the entry of microbes into the lamina propria. IECs consist of various specialized cells which differentiate from intestinal stem cells within the crypts. Enterocytes, the major components of IECs, are involved in nutrient absorption and transcytosis. Microfold cells (M cells) mediated antigens delivery from lumen to lymphoid tissues. Enteroendocrine cells produce hormones in regulating luminal sensing and secretions. Goblet cells and Paneth cells provide passive protection by secreting mucins and antimicrobial proteins, respectively [77].

GALTs are a collection of lymphoid tissues where antigens are taken up and processed. GALTs play critical roles in mediating the inflammatory response and immune tolerance. GALTs consist of both organized structures, such as mesenteric lymph nodes (MLNs), Peyer's patch, and the dispersed isolated lymphoid follicles (ILFs) which are enriched in lymphocytes, dendritic cells, and macrophages. The activation of mucosal immunity begins with antigen uptake by M cells from intestinal lumen to Peyer's patch where the antigens are further processed by antigen-presenting cells (APCs), and presented to naive T and B cells. Lymphocytes then migrate via afferent lymphatic vessels to the MLNs, where T cells and B cells mature and differentiate. Finally, activated lymphocytes enter the systemic circulation and travel back to lamina propria triggering antigen-specific immune responses [76]. The proper cooperation between IECs and GALTs contributes to intestinal homeostasis. Specifically, the homeostasis is accomplished through the secretion of mucus and antimicrobial proteins, and by the activation of both innate and adaptive immunity. Disruption in the balance of mucosal tolerance yield allergies, autoimmune diseases, and chronic inflammatory disorders [78].

2.2 Mucus layer

The intestinal epithelium is covered with a viscous gel-like layer of mucus. This mucus layer consists of abundant mucins glycoproteins, antimicrobial peptides, and immunoglobulins (IgA) which work together to maintain intestinal homeostasis. Mucus is continuous along the GI tract and organized in two distinct layers. The loosely adherent outer layer represents the

first-line of defense against the external environment. The inner-stratified mucus layer, firmly adherent to epithelial cells, is resistant to bacterial penetration. Both the secreted and adherent mucus layer continually renew across the GI tract, thereby inhibiting pathogens adherence and assisting pathogens clearance [79].

Mucins, secreted by Goblet cells, are the major components of mucus. To date, twenty-one mucin genes have been identified in the human genome and can be divided into secreted mucins and membrane-bound mucins [80]. Secreted mucins contribute to the elastic properties and assemble to a web-like structure of mucus gel. Membrane-bound mucins are expressed on the apical membrane of IECs and contribute to the viscous properties of mucus. Together, mucins create a physical and chemical barrier to prevent the direct contact of bacteria and toxins from the host tissues. Therefore, detection of bacteria in proximity of epithelial cells is a sign of impaired mucosal barrier, which can lead to bacterial translocation and subsequent inflammation [81-82]. For instance, MUC2-deficient mice are susceptible to colitis resulting from a direct contact of IECs with intestinal bacteria [83]. Decreased production of MUC2 has also been documented in patients with ulcerative colitis [84].

The extensive glycosylation of mucins confers protection from proteolytic digestion. Carbohydrates represent more than 80% of the molecular mass of mucins [85]. These glycans are clustered into O-glycosylation domains of proteins rich in repeating serine and threonine. In addition to O-glycans, mucins carry a relative minor amount of N-linked glycans [81]. The common O-glycans on mucins are summarized in **Figure 4**. Sulfation and sialylation are common terminal modifications that contribute to the highly negative charge of mucins. The terminal O-glycans of mucins are highly heterogeneous and vary between species and tissue locations. For instance, human intestinal mucins display an increasing gradient of sialic acid and a decreasing gradient of fucose across ileum to colon [86]. Moreover, altered mucin glycosylation has been associated with bacterial infection and various disease progression [87]. Impaired O-glycosylation on core 1, core 2, and core 3 structures **(Figure 4)** of intestinal mucins increase the susceptibility of mice to colitis development [88-90].

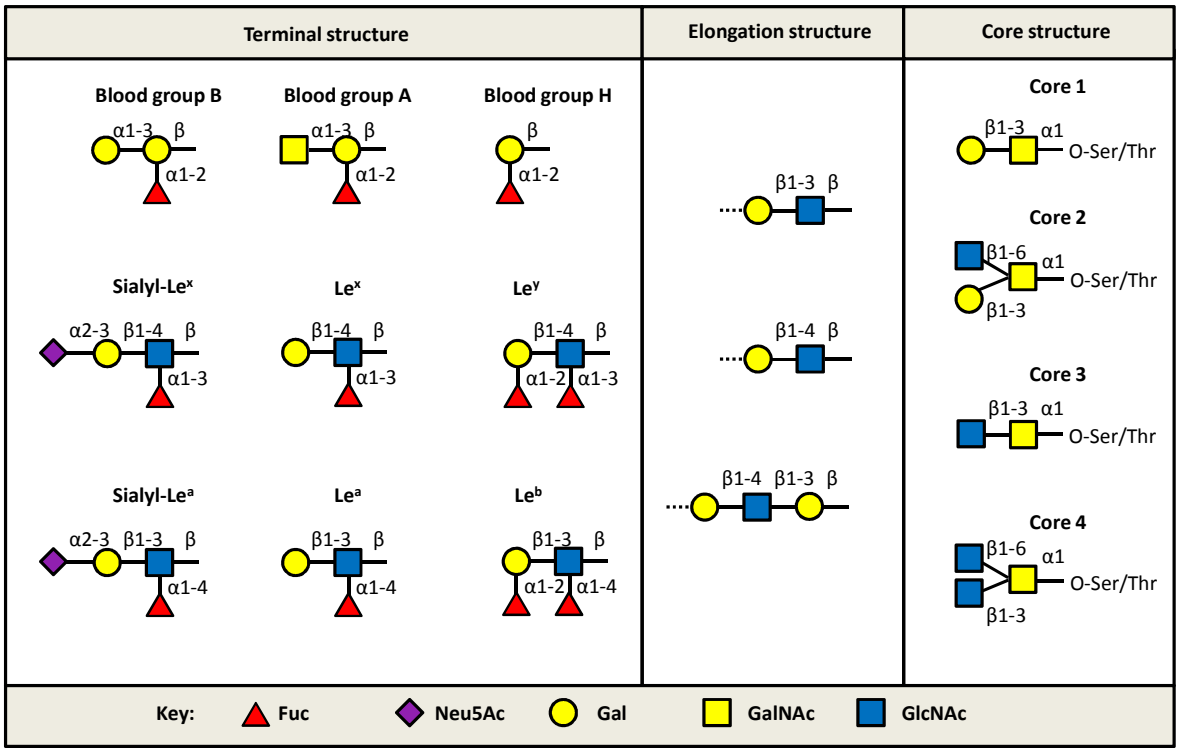


Figure 4. O-linked glycans on mucins (Adapted from [81]). O-linked glycosylation of mucins was initiated by the addition of *N*-acetyl-galactosamine (GalNAc) to the hydroxyl group of serine and threonine. GalNAc residue can be extended with Gal and GlcNAc and form 4 common core structures. These core structures can be further elongated with variable *N*-acetyllactosamine (LacNAc) chains, and terminated by Gal, Fuc or Neu5Ac. The terminal structures form histo-blood group antigens (A, B, H) and Lewis antigens (Le^a, Le^b, Le^x, Le^y) Fuc, fucose; Neu5Ac, *N*-acetylneuraminic acid; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine.

2.3 Antimicrobial proteins

A second immune component contributing to the intestinal barrier are antimicrobial proteins (AMPs) secreted by IECs and immune cells. AMPs function as effectors of innate immunity to directly kill or inhibit the growth of microorganisms in the gut mucosa. The characteristics of major AMP families in the intestine are summarized in **Table 3**. Most AMPs target the microbial cell wall and cell membrane. For instance, lysozyme kills bacteria through enzymatic attack on the cell wall by hydrolyzing the glycosidic linkages of peptidoglycans [91]. Phospholipase A2 kills bacteria by hydrolyzing bacterial membrane phospholipids [92]. Other AMPs, such as lipocalin and calprotectin possess high metal chelating properties, depriving

essential nutrients of bacteria from the environment, thereby limiting bacterial growth in the gut mucosa [91].

Defensins are cysteine-rich cationic proteins representing the major family of AMPs. Defensins are classified into α- and β-defensins according to the distribution of cysteines and disulfide bonds. Human α-defensins include human neutrophil peptides (HNP1-4), which are mainly secreted by neutrophils in the lamina propria [93], and HD5-6, which are expressed by Paneth cells. Human β-defensins (hBD1) are constitutively expressed by colonic epithelial cells. By contrast, hBD2 to 4 are secreted in response to external stimuli including various microorganisms and cytokines [94]. For instance, the induction of hBD2 is mediated by proinflammatory cytokines including IL-1β and TNF-α [95]. Among the defensins, hBD3 displays a potent and broad spectrum of antimicrobial activity because of its high positive net charge [96], whereas hBD1 exhibits enhancing antimicrobial activity after reduction of disulfide bonds in anaerobic condition [97]. Besides antimicrobial activity, defensins have additional immunomodulatory activities as chemoattractants for leukocytes recruitment to the site of infection [98]. For instance, hBD1-3 facilitate the recruitment of memory T cells and immature DCs [99] while hBD3 and hBD4 mediate monocytes chemotaxis [100-101]. Additionally, defensins stimulate the proliferation of intestinal epithelial cells, and neutralize lipopolysaccharide (LPS) by preventing its oligomerization and receptor binding [102].

Overall, the multiple functions of AMPs are essential for intestinal homeostasis. As a result, impaired production of AMPs is associated with intestinal dysbiosis, susceptibility to pathogenic infections and multiple inflammatory disorders [91, 103].

Table 3. Major antimicrobial protein families in the intestine (Adapted from [91])**.**

Family	proteins	Mechanism of action	Tissue expression	Cellular expression
α-defensin	HD5-6 (in humans) Cryptdins (in mice)	Membrane disruption	Small intestine	Paneth cells, neutrophils, macrophages
β-defensin	BD1-3	Membrane disruption; lipid II binding	Large intestine,	Enterocytes
Calprotectin		Metal chelation	Abscesses	Neutrophils
Cathelicidin	LL37 (in human) CRAMP (in mice)	Membrane disruption	Large intestine	Neutrophils, mast cells
C-type lectin	HIP/PAP (in human) REG3γ (in mice)	Peptidoglycan recognition	Small Intestine	Paneth cells, enterocytes
Galectin	GAL4, GAL8	Unknown	Intestine	Epithelial cells
Lipocalin	Lipocalin 2	Iron chelation	Intestine	Macrophages, epithelial cells
Lysozyme		Hydrolysis of cell wall peptidoglycan	Intestine secretions	Paneth cells
Phospho-lipase A2		Hydrolysis of membrane phospholipids	Intestine secretions	Paneth cells, macrophages

BD, β-defensin; CRAMP, Cathelicidin-related antimicrobial peptide; GAL, galectin; HIP/PAP, hepatointestinal pancreatic/pancreatitis-associated protein; REG3γ, regenerating islet-derived protein 3γ

2.4 Innate immunity

Innate immunity is the first response to acute inflammation and also plays a regulatory role in adaptive immunity [104]. Innate immune cells consist of NK cells, mast cells, granulocytes (neutrophils, eosinophils, and basophils), and mainly DCs and macrophages in the gut mucosa. Neutrophils are the most abundant type of phagocytes, representing about 50% of circulating leukocytes, and are the first wave of cells to arrive to the sites of infection. They contribute to the fast clearance of pathogens by phagocytosis and release of granular proteins, but also secrete cytokines (IL-1, IL-6, IL-12, TNF-a) and chemokines which recruit and activate other immune cells. DCs have been characterized into a diverse subset based on the differential expression of cell markers (CD11b, CD11c, CD103) and chemokines receptors

(CCR7, CX3CR1). DCs exhibit phagocytic activity and antigen presenting activity, hence promoting the differentiation and maturation of lymphocytes into effector cells. Intestinal DCs are also responsible for inducing immune tolerance to commensal bacteria through induction of regulatory T cells and IgA-secreting B cells [105]. Macrophages present in lamina propria also exhibit high phagocytic and bactericidal activity through production of AMPs and reactive oxygen species [106]. Macrophages produce large amounts of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α that recruit neutrophils and activate T cells during inflammation. By contrast, under normal conditions, intestinal macrophages produce anti-inflammatory cytokines such as IL-10, TGF-β, and retinoic acid promoting intestinal homeostasis [107].

The ability of innate immune cells to distinguish self- from non-self antigens relies on the expression of pattern recognition receptors (PRRs). PRRs are responsible for sensing the distinct microbial molecules, termed pathogen-associated molecular patterns (PAMPs), and the endogenous molecules released from damaged tissues, termed damage-associated molecular patterns (DAMPs) [108]. PAMPs are components specifically found in microbes, such as modified nucleic acids (bacterial or viral DNA and RNA), proteins (flagellin, profilin, envelope proteins), carbohydrates (LPS, zymosan, peptidoglycans) and lipoproteins (diacyl-, triacyl-lipoprotein). Because PAMPs are essential for the survival of microbes, these highly conserved molecular patterns represent ideal targets for innate immune cells. Exposure of PRRs to their ligands activates intracellular signaling cascades in immune cells, which induce a variety of immune responses aiming at the elimination of pathogens. Thus far, PRRs are divided into secreted, cytoplasmic, and transmembrane receptors based on their cellular localization. The major groups of PRRs and their microbial ligands are summarized in **Table 4**.

(1) Secreted PRRs include collectins, ficolins, pentraxins, and complement receptors. These receptors are involved in the activation of complement system, microbial opsonization, and phagocytosis. For instance, collectin and pentraxin can bind to a wide range of microbial carbohydrate epitopes or membrane phosphocholine, and then lead to the activation of complement system and phagocytosis [109-110].

(2) Cytoplasmic PRRs include the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs) **(Table 4)**. Intracellular RLRs act as sensors during viral replication by recognizing double-stranded RNA (dsRNA) from dsRNA viruses or from the replication intermediate of single-stranded RNA viruses. Upon ligand binding, RLRs can activate the expression of type I IFN and inflammatory cytokines [111]. NLRs are composed of two major families, including nucleotide-binding oligomerization domain (NOD) receptors and NLRP family (NLRP1-14). NOD1 and NOD2 recognize the components of bacterial peptidoglycan, such as mesodiaminopimelic acid and muramyl dipeptide, respectively, which lead to transcriptional activation of proinflammatory cytokine via NF-κB pathway [112]. Other NLRs involve in the formation of the inflammasome and mediate caspase-activation and IL-1 secretion [113].

(3) Transmembraneous PRRs include C-type lectin receptors (CLRs) and Toll-like receptors (TLRs). CLRs recognize microbial surface carbohydrates in a calcium-dependent manner. For instance, dectin-1 and dectin-2 are transmembrane proteins of CLRs with an extracellular carbohydrate recognition domain (CRD) and immunoreceptor tyrosine-based activation motif (ITAM)-like intracellular tail [114]. Dectin-1 and dectin-2 which expressed by DCs, macrophages, and neutrophils, are responsible for sensing β-glucans and α-mannan from fungi, respectively [115-116]. Upon ligand binding, SH2 domain-containing tyrosine kinases are recruited to ITAM, following by transcriptional activation of cytokines genes such as IL-1β, IL-12 and IL-23 in response to fungal infection.

TLRs compose the major groups of transmembrane PRRs. TLRs are induced or constitutively expressed throughout the GI tract by a variety of cells, including IEC lineages, DCs, and macrophages. To date, thirteen TLRs have been identified in mammals, but only ten have been reported in human. Based on the ligand recognition sites, TLRs are localized either at the cell surface or in intracellular (endosomal/lysosomal) compartment **(Table 4)**. TLRs consist of extracellular leucine-rich repeats (LRRs) involved in ligand binding, a transmembrane domain, and a cytoplasmic Toll/IL-1R homology (TIR) domain involved in intracellular signaling [108]. The TLRs signaling pathways are depicted in **Figure 5**.

Table 4. Pattern recognition receptors and their microbial ligands (Adapted from [104, 108])

TLR	Localization	Ligand	Origin of the ligand
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria and mycobacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria and mycobacteria
		peptidoglycan	Gram-positive bacteria
		Phospholipomannan	Fungi
		Zymosan	Yeast
TLR3	Endolysosome	dsRNA	Viruses
TLR4	Plasma membrane	LPS	Gram-negative bacteria
		Mannan	Fungi (*Candida* spp.)
		Envelope proteins	Viruses
TLR5	Plasma membrane	Flagellin	Flagellated bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Mycobacteria
		Lipoteichoic acid	Gram-positive bacteria
		Zymosan	Fungi
TLR7/8	Endolysosome	ssRNA	RNA Viruses
TLR9	Endolysosome	CpG-DNA (unmethylated)	Bacteria
		DNA	Viruses
		Hemozoin	Protozoa (*Plasmodium*)
TLR10	Plasma membrane	Unknown	Unknown
TLR11/12	Endolysosome	Profilin-like molecule	Protozoa (*Toxoplasma)*
TLR13	Endolysosome	23S rRNA	Bacteria
RLR			
RIG-1	Cytoplasm	dsRNA (5’ triphosphate)	RNA viruses, DNA viruses
MDA5	Cytoplasm	Long dsRNA	RNA viruses
NLR			
NOD-1	Cytoplasm	Peptidoglycan (iE-DAP)	Bacteria
NOD-2	Cytoplasm	Peptidoglycan (MDP)	Bacteria
CLR			
Dectin-1	Plasma membrane	β -glucan	Fungi
Dectin-2	Plasma membrane	β –glucan and α-mannan	Fungi

MDA5, Melanoma differentiation-associated gene 5; MDP, muramyl dipeptide; iE-DAP, g-D-glutamyl-mesodiaminopimelic acid;

TLR signaling is essential to initiate the innate immune response against pathogens. Consequently, a defective TLRs signaling is associated with various diseases. For instance, TLR5-deficient mice develop spontaneous colitis [117]. TLR2- and TL4-deficient mice are susceptible to bacterial infections [118], and TLR11-deficient mice are susceptible to intracellular parasite infection [119]. Moreover, aberrant activation of TLRs is also involved in the pathogenesis of autoimmune and chronic inflammatory diseases [120]. To limit inappropriate immune activation to commensal bacteria, TLR signaling is tightly regulated by restricted expression patterns or functional compartmentalization. For instance, bone marrow-derived DCs express high levels of TLR4 to rapidly recognize detrimental threats in the blood circulation, whereas LP-derived DCs express low levels of TLR4 to avoid hyper-responsiveness against widespread LPS in the gut lumen [121]. TLR5, the receptor for bacterial flagellin, is mainly localized at the basolateral surface of IECs. This expression pattern ensures that immune activation only occurs during bacterial invasion as a result of impaired epithelial junctions, but not over activates at the apical mucosal surface.

Additional mechanisms that negatively regulate TLR signaling, include soluble decoy receptors, intracellular and membrane-bound inhibitors [122]. Soluble decoy TLRs (sTLR2 and sTLR4) function as antagonists, and prevent over-activation of TLR signaling [25, 123]. Intracellular regulators like suppressor of cytokine signaling 1 (SOCS1) and IL-1 receptor-associated kinase-M (IRAKM) down-regulate TLR signaling through inhibition of IRAK-TRAF6 complex [124-125]. Membrane-bound inhibitors include ST2, single immunoglobulin IL-1-related receptor (SIGIRR), and TNF-related apoptosis-inducing ligand receptor (TRAILR). ST2 inhibit TLR signaling by sequestration of the adaptor MyD88 and MAL proteins [126]. SIGIRR binds to TLR4 and IRAK and suppresses downstream signaling [127], whereas TRAILR suppress NF-κB activation in both MyD88-dependent and independent pathways [128]. Overall, negative regulation is required to balance immune responses, since defects of negative regulators are associated with autoimmune, chronic inflammatory and infectious disease [122, 129].

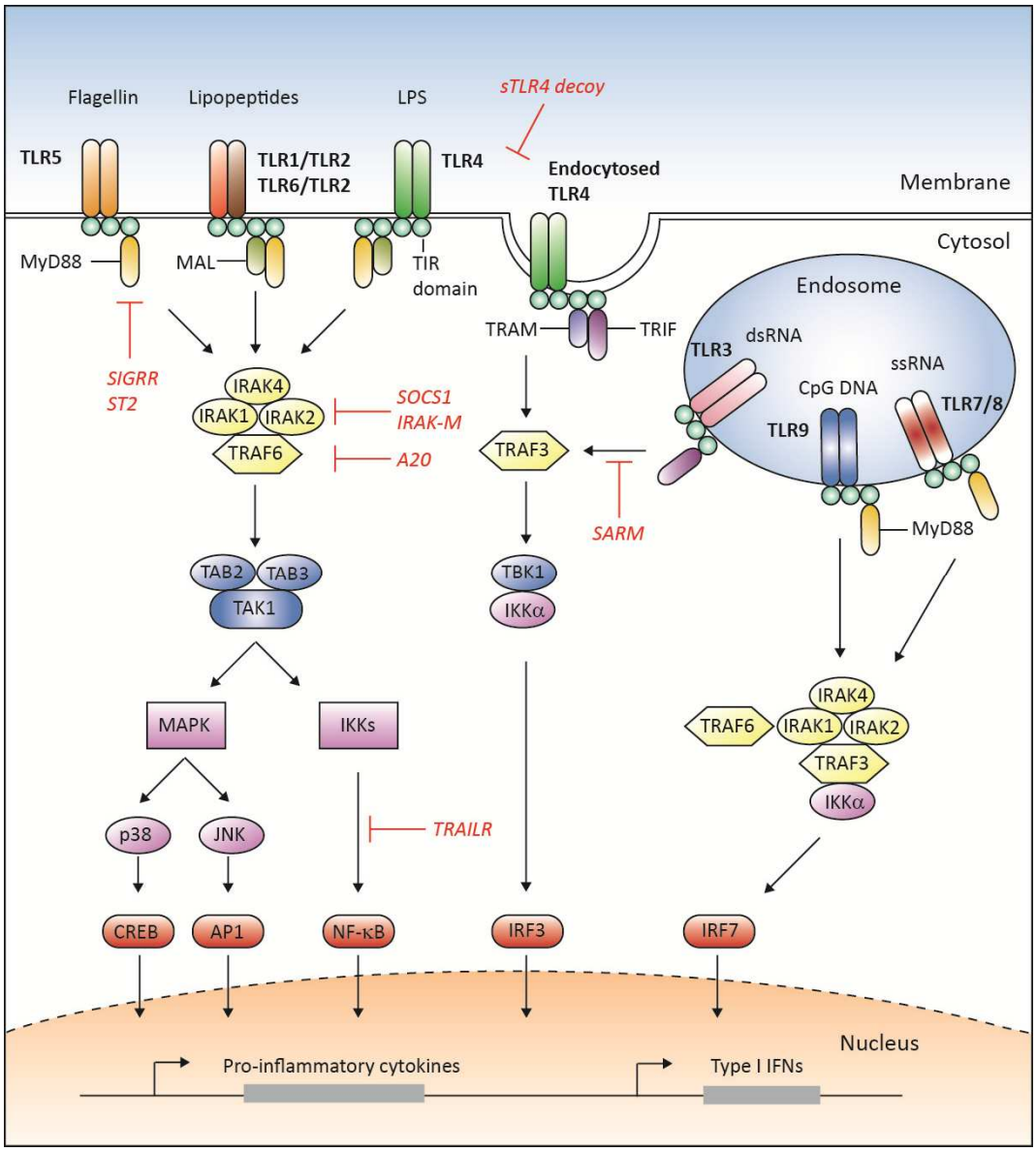


Figure 5. Toll-like receptor signaling pathways (Adapted from [122, 130]). TLRs signaling is initiated by ligand-induced dimerization of receptors, either in plasma membrane or endosome, followed by activation of MyD88-dependent or TRIF-dependent pathway. Engagement of adaptor proteins to TIR domain activates IRAKs and the adaptor molecules TNF receptor-associated factors (TRAFs), and lead to transcriptional factors such as NF-κB and IRF3. Hence, the transcriptional activation leads to the expression of pro-inflammatory cytokines or Type I IFN. IRAK, IL-1R associated kinase; IRF, Interferon-regulatory factor; IKK, inhibitor of NF-κB; MAPK, mitogen-activated protein kinase; TRAILR, TNF-related apoptosis-inducing ligand receptor; TAB, TAK-1 binding protein; TAK, TGFβ-activated kinase; MyD88, myeloid differentiation primary response protein 88.

2.5 Adaptive immunity

The efficient clearance of pathogens and induction of tolerance to commensal bacteria at intestinal mucosa requires the coordination of innate and adaptive immune system. Upon antigenic stimulation, innate immunity triggers the activation of adaptive immunity. In contrast to innate immunity, adaptive immunity confers highly specific and long-lasting immunity. Adaptive immune cells consist of a repertoire of T and B lymphocytes, whose differentiation is coordinated by antigen-presenting cells (APCs), cytokines, and other environmental factors. APCs play a critical role in the crosstalk between innate and adaptive immune responses.

Naive T cells are divided into $CD4^+$ T-helper (T_H) cells and $CD8^+$ cytotoxic T cells (CTLs). T_H cells are activated through the binding of antigens presented on MHC-II by APCs, whereas CTLs are activated through MHC-I found on most nucleated cells. Upon activation, T_H cells respond to various cytokine-signals and differentiate into several subtypes, such as T_H1, T_H2, and T_H17 cells, which promote inflammation and clearance of pathogens, or regulatory T cells (T_{Reg} or T_R1), which mediate immunosuppression. The differentiation of intestinal $CD4^+$ T cells are summarized in **Figure 6**.

T_H cells are characterized by the production of distinct cytokines and the expression of lineage-specific transcription factors. T_H1 cells elicit cell-mediated immunity and inflammation against mainly intracellular bacterial and viral antigens. Upon antigenic stimulation, naive T cells primed in the presence of IL-12, differentiate into T_H1 cells and induce the production of cytokines such as IL-2, IFN-γ, and TNF-α. T_H1 cytokines enable the activation and recruitment of macrophages, NK cells, and CTLs to the site of infection. By contrast, T_H2 cells are involved in humoral-mediated immunity against the extracellular parasites. After activation in the presence IL-4, naive T cells differentiate into T_H2 cells, and secrete themselves IL-4, IL-5, and IL-13. Consequently, the induction of T_H2 cytokines triggers the production of IgE from B cells and promote eosinophils-mediated inflammation against the extracellular parasites. Notably, key cytokines (e.g. IFN-γ, IL-4) produced from T_H1 and T_H2 cells, and lineage-specific transcription factors (e.g. T-bet, GATA-3) provide positive

feedback mechanisms to maintain self-differentiation, but also mutually inhibit each others to reinforce their lineage fate.

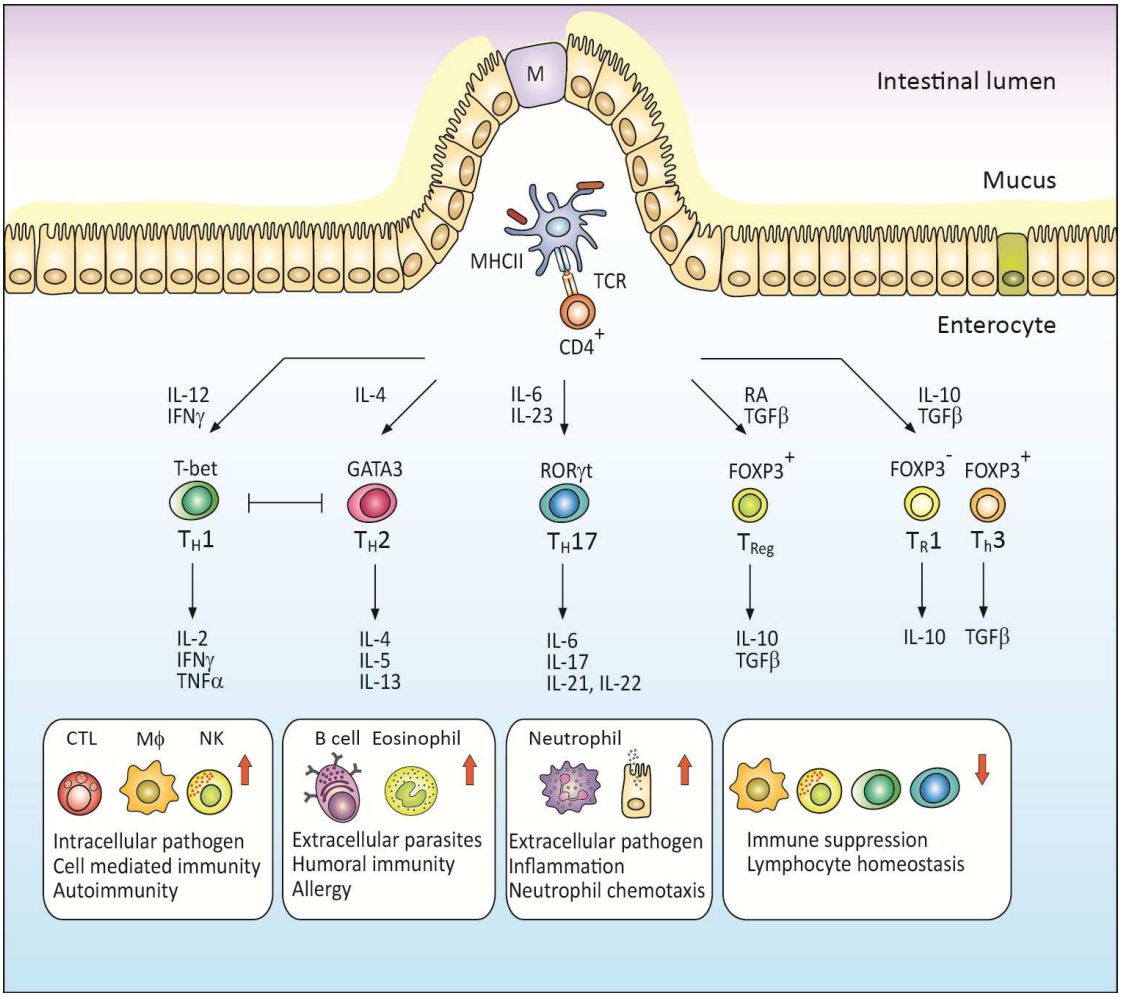


Figure 6. Differentiation of intestinal CD4$^+$ T cells (adapted from [131-132]). Differentiation of T_H cell subsets from naive T cells is induced upon interaction with the antigen associated MHC-II and co-stimulatory signal by antigen presenting cells. T_H cells can differentiate into effector T cells (T_H1, T_H2, T_H17) that promote inflammation and clearance of pathogens, or regulatory T cells (T_{Reg}, T_R1, and T_h3) that suppress immune response. Differentiation of T_H cells is mediated by cytokine-induced signals (such as IL-4, IL-6, IL-10, IL-12, and TGF-β) and the expression of transcription factors (T-bet, GATA-3, RORγt and FOXP3). CTL, cytotoxic T cells; MΦ, macrophage; NK, natural killer; RA, retinoic acid; T-bet, T-box transcription factor; GATA3, GATA-binding protein 3; RORγt, RAR-related orphan receptor γ; FOXP3, Forkhead box P3.

The discovery of T_H17 cells has opened a new concept to the T_H1/ T_H2 paradigm of adaptive immunity. T_H17 cells exhibit distinct effector functions than T_H1 and T_H2 cells, mediating the clearance of extracellular pathogens like bacteria and fungi. Upon antigenic stimulation, T_H17 cells, activated by IL-6 and IL-23, are responsible for producing IL-17A and IL17F which promotes neutrophil chemotaxis and pathogen elimination. T_H17 cells can also produce IL-22, which contributes to maintaining the epithelial barrier by enhancing the secretion of antimicrobial proteins and mucins from IECs [133]. Defects in T_H17 cell functions increase susceptibility to commensal fungal infections, as shown by the reduction of IL-22 and neutrophils recruitment in both T_H17 (*Il23p19*$^{-/-}$) and IL-17R (*Il17r*$^{-/-}$) deficient mice, demonstrating the importance of T_H17 mediated anti-fungal response in mucosal immunity [134]. However, aberrant regulation of T_H17 also leads to chronic intestinal inflammation [135-136] and contributes to the emergence of autoimmune diseases [137-138].

The balance between immune activation and immune tolerance is controlled by a specialized T cell subset, known as T_{Reg}. The differentiation of functional T_{Reg} is activated by retinoic acid, TGF-β and lineage-specific transcription factor forkhead box P3 (FOXP3). Deficiency in FOXP3 is associated with massive lymphocytes proliferative disease [139]. FOXP3$^+$-T_{Reg} cells produce large amounts of TGF-β, IL-10, and IL-35 which suppress the expansion of effector T_H1 and T_H17 cells, and also block the activation of macrophages and NK cells in the intestine [140].

The critical roles of T_{Reg} in mediating immunosuppression are manifested by the secretion of anti-inflammatory cytokines, such as IL-10 and TGF-β. These cytokines are essential for intestinal homeostasis as shown by the spontaneous intestinal inflammation characterizing mice deficient of IL-10 and TGF-β [141-142]. Moreover, mutations of IL-10 receptor are also associated with inflammatory bowel disease in human [143]. Apart from natural T_{Reg}, another subset of regulatory T cells, known as T_R1 and T_h3 cells, can be induced in the periphery. These cells are important in maintaining immune-tolerance mainly through the production of high level of IL-10 and TGF-β, respectively [144]. Along with T-cell mediated immunity, the humoral response induced by B-cells contributes to protecting the host from pathogenic infections. The activation of B cells is mainly driven by T_H2-cytokines, such as IL-4 and IL-5, but can also be induced by DC-driven responses. Upon antigenic stimulation,

mucosal B cells differentiate into plasma B cells and secrete IgA. IgA is the most abundant immunoglobulin isotype in the intestinal mucosa. Secretory IgA is transported through the epithelial layer (transcytosis) to the intestinal lumen and opsonized bacteria or toxins to restrict their attachment and penetration of the gut epithelial layer [145]. In addition, study on IgA-deficient mice suggested the importance of sIgA to block virus entry into Peyer's patches [146]. TGF-β signaling is also essential for the induction of IgA [147]. Besides the prevention of pathogenic infection, sIgA also exhibits anti-inflammatory effects, contributing to the mucosal homeostasis.

3. Gut microbiota and immune system

The human GI tract is colonized by large numbers of diverse microorganisms, including fungi, parasites, virus, and mostly bacteria, which is collectively referred to as the gut microbiota. The heterogeneous microenvironment in the gut influences the abundance, diversity, and distribution of the gut microbiota through the GI tract. Bacterial numbers can range from 10^2-10^3 per gram luminal contents in gastric-duodenal region to 10^{11}-10^{12}/g predominantly anaerobic bacteria in the caecum and distal colon [148]. It is generally accepted that the intestinal microbiota comprises more than 1000 different bacterial species [149], however only a small minority can be cultured. Recently, advances in culture-independent sequencing have greatly improved our understanding to a variety of microbes and enabling the rapid detection of spatial, temporal, and compositional changes in our microbiota. In human, the dominant gut bacterial species belong to two major phyla, Firmicutes and Bacteroidetes (>90%) whereas Actinobacteria, Proteobacteria Cyanobacteria, Fusobacteria and Verrucomicrobia present in relatively low abundance [150]. Moreover, a comprehensive study of global gut microbiota has categorized the human microbiota into three predominant enterotypes (Bacteroides, Prevotella, or Ruminococcus) as driven by dietary intake [151].

3.1 Contributions of the gut microbiota

The colonization of the GI tract by commensal microbiota leads to a mutualistic relationship between host and microbiota. The investigation on germ-free (GF) mice outlines the multiple roles of microbiota in the host physiology including metabolic, protective, and structural functions **(Figure 7)**. The gut microbiota supports normal metabolic functions of the host by degradation of non-digestible substances, production of short-chain fatty acids (SCFAs) and essential vitamins, and absorption of ion. This is evidenced in GF animals, which require a higher caloric intake [152], and are prone to vitamin deficiencies [153] compared to conventionally reared animals. It is also known that microbiota is pivotal in regulating intestinal morphology, as shown in GF animals which have aberrant crypt and villus [154], impaired IECs proliferation and reduced epithelial tight junctions [155]. Notably, GF animals are more susceptible to infection due to multiple defects in the mucosal immunity. These

include a reduced size of MLNs and Peyer's patch follicles [156], decreased plasma cells and IgA production [157], decreased Paneth cells and antimicrobial proteins [158], and decreased isolated lymphoid follicles [159]. However, reconstitution of commensal bacteria is sufficient to provide protection against infections implying that gut microbiota is critical to restore the intestinal barrier and mucosal immunity [160-162]. Moreover, components derived from commensal bacteria such as LPS and polysaccharide A (PSA) can also affect the normal development of mucosal immunity [163].

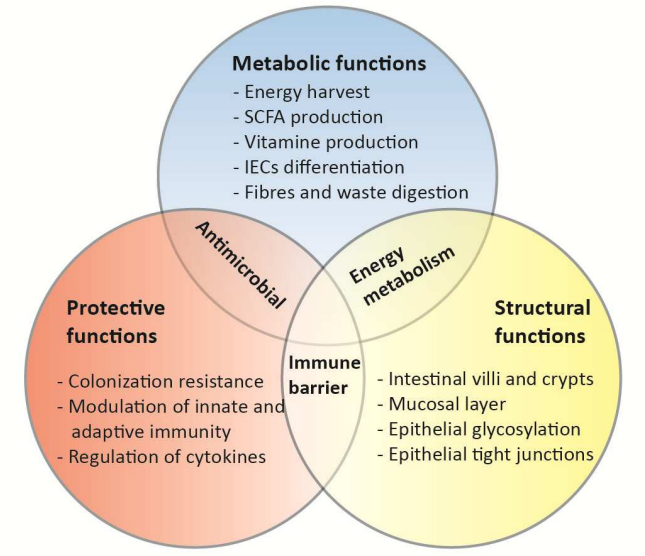


Figure 7. Functions of the intestinal microbiota (adapted from [154, 164]). Commensal bacteria display multiple biological functions on the intestinal mucosa of the host.

3.2 Gut microbiota and IBD

The composition of the human gut microbiota is highly dynamic at the infant stage. During infancy, external factors, such as delivery mode and feeding, initially influence the colonization of gut microbiota. As the infant grows, diet, environmental and genetic factors progressively shape the gut microbiota and toward a relatively stable microbial community in adult. Attempts to characterize the bacterial shift between healthy and disease state provide us a first picture to predict the disease progression and to develop relevant therapies. The imbalance of microbiota composition is referred to as dysbiosis, and has been associated with various disorders such as allergy, autism, obesity, diabetes, inflammatory bowel disease

(IBD), and cancer [165]. The impact of the microbiota on human health is well characterized by studies on IBD. IBD is primarily associated with the degree of industrialization, as observed from the highest incidence rate in Europe and North America. The major clinical forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC). Both are chronic and relapsing inflammatory disorders of the gastrointestinal tract. Of which, CD affects the entire GI tract and is characterized by transmural inflammation with large ulcerations and granulomas formation, whereas, UC mainly affects on colon and rectum with superficial ulceration and crypt abscesses [166]. Although the exact etiology of IBD remains unclear, it is generally hypothesized that the disease developed in genetically predisposed individual who has overactive immune responses against the commensal bacteria [167]. Recently, genome-wide association studies have been successfully identified genetic variants that contribute to IBD susceptibility [168-169]. Despite distinct clinical patterns, approximately 30% of IBD-related genetic abnormalities are shared between CD and UC [170], and are categorized into epithelial barrier functions (CDH1, HNF4a, PTPN2), innate sensors recognition (NOD2, CARD9, TLR4), immune cells regulation (STAT3, IL-2, IL-10, IL-23R), ER stress (XBP1) and autophagy (ATG16L1) **(Figure 8)**.

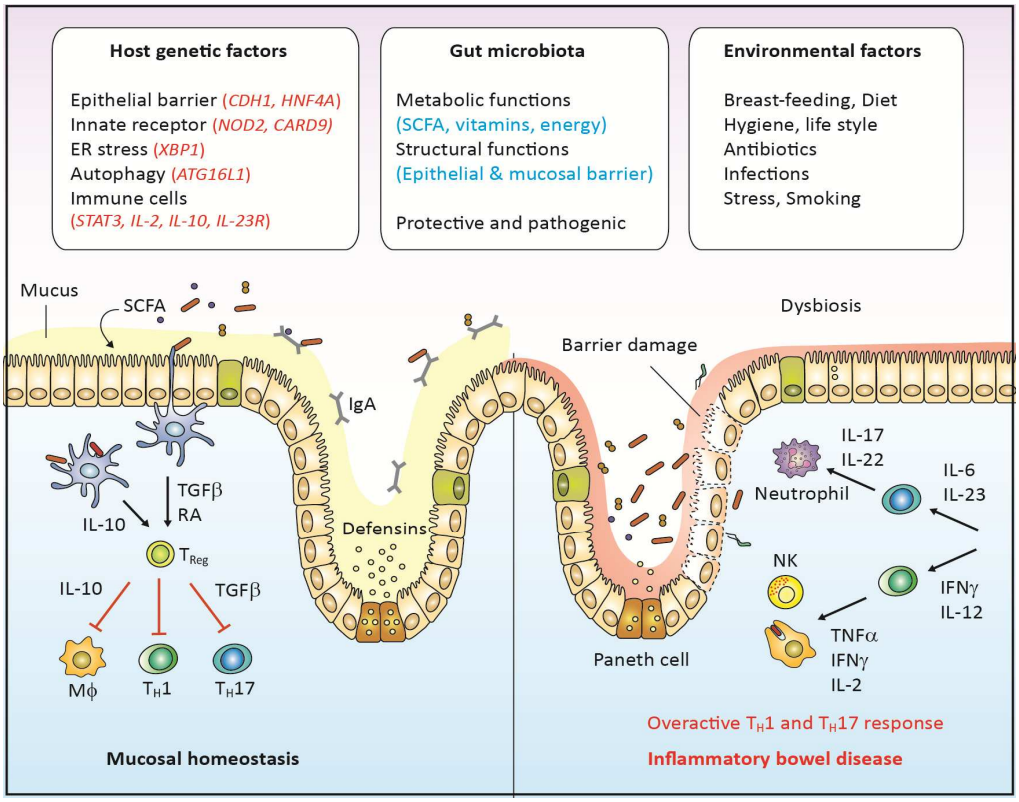


Figure 8. Development and pathogenesis of IBD (adapted from [171]). Host genetic and environmental factors influence the microbial homeostasis in the intestine mucosa. In homeostasis, commensal bacteria trigger tolerogenic DCs and the development of regulatory T cells. The development of IBD is frequently initiated by the altered barrier function, translocation of bacteria into tissue, and subsequent immune activation in genetically susceptible individuals. Failure of immune regulation further leads to chronic inflammation, dysbiosis and irreversible tissue damage which are driven by cytokine responses.

The first identified gene correlating with human IBD was NOD2. NOD2 is an innate receptor, which recognizes bacterially derived muramyl dipeptide and triggers both innate and adaptive immune responses. Studies based on *Nod2*-deficient mice and CD-associated *Nod2* mutations in human suggest the defects of such innate sensor results in decreasing defensins production by Paneth cells, and reducing proinflammatory cytokines production (IL-1β, TNF-α, IL-6 and IL-23) by antigen-presenting cells. Other families of innate receptors such as NLRs and RLRs also associated with innate defense, as evidenced by an increased susceptibility to colitis in NLRP3 or RIG-I deficient mice [172-173]. Adaptor protein like CARD9 (Caspase recruitment domain-containing protein 9) integrates the signals from innate receptors to downstream NF-κB signaling is also implicated in IBD [174]. In addition to innate defense, genes associated with epithelial tight junction including *CDH1* (E-cadherin) and *PTPN2* (protein tyrosine phosphatase) are critical in maintaining intestinal barrier, also implicated in human IBD [175-176]. Moreover, mutations in the autophagy regulatory genes (*ATG16L1*) and immunity-related GTPase family M (*IRGM*) are also observed in CD patients due to abnormalities of Paneth cell and defects in bacterial clearance [177-178].

The onset and relapse of IBD are also associated with environmental factors which affect the intestinal mucosa barrier or immune responses. These factors include hygiene, diet, medication, infection, stress, and smoking [179]. However, how these factors initiate or reactivate the disease remain poorly understood. Medication such as antibiotics and nonsteroidal anti-inflammatory drugs (NSAIDs) influence mucosal barrier and mediate non-specific inflammation, as evidenced by a significant correlation between antibiotic use and CD [180], as well as NSAIDs-induced colonic ulcerations [181]. Stress is also implicated in the intestinal inflammation due to an increasing epithelial permeability and mucosa damage

in animal models [182-183]. Notably, smoking is well implicated in IBD pathogenesis, however, plays a paradoxical role in CD and UC patients. A meta-analysis has suggested that active smokers increase the risk of developing CD but decrease the risk of developing UC compared to non-smokers [184]. The impact of diet on IBD has also been extensively studies. A systemic review has suggested that high dietary intakes of total fats and PUFAs are positive correlated with an increased risk of both CD and UC [185].

3.3 Pathogenic role of gut microbiota in IBD

Alteration of microbiota composition has long been observed in human IBD and experimental colitis. Numerous studies have indicated a potential pathogenic role of gut microbiota in the progression of the disease, as evidenced by amelioration of colitis in antibiotic-treated and GF animals. Commensal bacteria are critical to maintain the gut homeostasis and symbiotic relationship between the host. However, some bacteria can acquire pathogenic features under abnormal conditions, such as acute inflammation and dysregulation of the immune system. These bacteria are collectively referred to as pathobionts, such as *E. coli*, *Enterococcus faecalis*, *Bacteroides vulgatus*, *Prevotella* and *Klebsiella* species which have all been shown to induce colitis in genetic predisposed mouse [186] **(Table 5)**. The colitogenic property of pathobionts nevertheless depends on the host specific immune defects, as evidenced for *B. vulgatus* that triggers colitis in *Il10r2*$^{-/-}$ and *Tgfbr2*$^{-/-}$ mice but not in *Il2*$^{-/-}$ or *Il10*$^{-/-}$ mice [187-188]. On the other hand, *E. coli* induces colitis in germ-free *Il10*$^{-/-}$ mice but not in *Il10r2*$^{-/-}$ and *Tgfbr2*$^{-/-}$ mice [187, 189].

Meta-analysis of microbial diversity has frequently observed an expansion of *Proteobacteria* in patients with IBD. In particular, increasing numbers of adherent invasive *E. coli* (AIEC) [190], *Campylobacter*, *Salmonella* and *Helicobacter* spp. have all been observed in patients with IBD **(Table 5)**. The mechanism by which AIEC induce colitis is associated with their invasiveness and potential to drive inflammatory responses. AIEC invades and survives within macrophage phagolysosome without inducing cell death, allowing them to continuously proliferate and trigger TNF-α release which then drives chronic inflammation [191]. AIEC has also been reported to induce the aggregation of infected macrophages and lymphocytes to form

granulomas, a clinical feature in CD patients [192]. Taken together, these virulence-like factors highlight the causative role of AIEC in the development of IBD.

Table 5. Gut microbiota associated with IBD pathogenesis (Adapted from [186, 193]).

Bacteria species	Host type	Disease model	Potential mechanisms
Adherent-invasive E. coli (AIEC)	Human	CD	Increased prevalence in patients, induce granulomas, increase TNF
Salmonella spp.	Human	UC and CD	Previous infection predisposes to IBD
Campylobacter jejuni	Human	UC and CD	Previous infection predisposes to IBD
Campylobacter concisus	Human	UC and CD	Increased IL-8 and TNF activation
*Helicobacter*spp. *(Enterohepatic)*	*Il10*$^{-/-}$ Mouse	Spontaneous colitis	Induced colitis in *Il10*$^{-/-}$ mice
Klebsiella spp.	Mouse	TNBS-induced colitis	Increased IL-1β, IL-6 and TNF-α activation
Prevotellaceae TM7	*Nlrp6*$^{-/-}$, *Asc*$^{-/-}$ *or Casp1*$^{-/-}$ *Mouse*	DSS-induced colitis	Impaired IL-18 signaling promotes pathobiont expansion
B. vulgatus *B. thetaiotaomicron*	*Il10r2*$^{-/-}$ Mouse *Tgfbr2*$^{-/-}$ Mouse	Spontaneous colitis	Colonization of antibiotic-treated mice triggers colitis
Escherichia coli	*Il2*$^{-/-}$ Mouse *Il10*$^{-/-}$ Mouse	Spontaneous colitis	Monocolonization of GF mice induces colitis
Enterococcus faecalis	*Il10*$^{-/-}$ Mouse	Spontaneous colitis	Monocolonization of GF mice induces colitis

DSS, dextan-sulfate sodium ; TNBS, 2,4,6-trinitrobenzene sulphonic acid; Il2, interleukin 2; Il10, interleukin 10; *Nlrp6,* NOD-, LRR- and pyrin domain containing 6 ; Casp1, caspase 1; Asc, apoptosis-associated speck-like protein containing-CARD; Tgfbr2, transforming growth factor beta receptor 2; TNF, tumor necrosis factor;

Increasing evidences also demonstrated that previous infection with a class of *Campylobacter*, *Salmonella,* or *Helicobacter* species increase the risk of the host to develop IBD [194]. For example, exposure of *C. jejuni* to host has been shown to induce murine colitis [195] and highly correlated with human IBD [196]. A high prevalence of *Campylobacter concisus* is also observed in IBD patients [197-198]. Characterization of *C. concisus* strain has revealed its ability to secrete cytolethal toxins and to induce inflammatory response. Studies have also shown that infection with *Helicobacter hepaticus* significantly exacerbate colitis in

Il10$^{-/-}$ mice through IFN-γ and IL-12 driven over-activation of T_H1 response [199]. Taken together, the development of IBD is associated with host specific immune factors and the resulting microbial dysbiosis. The proposed mechanisms by which the accumulation of these pathobionts influence the susceptibility of IBD are associated with bacterial toxins, breakage of epithelial barrier, increase of membrane permeability, and stimulation of T_H1 and T_H17 inflammatory responses [200].

3.4 Protective role of gut microbiota in IBD

Although various pathobionts promote the development of IBD, some commensal bacteria also contribute to lessening the severity of IBD. The protective role of commensal bacteria is demonstrated in GF mice, which are more susceptible to experimental colitis than conventionally reared mice, whereas reconstitution of commensal bacteria decrease the disease [201]. The beneficial roles of these probiotics to gut mucosa of the host are associated with their immunomodulating activities and metabolic functions **(Table 6)**. For example, reconstitution of *Clostridium* spp. (clusters XIVa and IV) has been shown to ameliorate the experimental colitis in mice through induction of FOXP3$^+$-T_{Reg} cells and the reduction of systemic IgE response [202]. In addition, numerous *Bifidobacteria* and *Lactobacillus* spp. have been shown to initiate T_{Reg} cells and mediate anti-inflammatory response in both murine colitis model and human IBD [203-204].

Commensal bacteria also exert anti-inflammatory effects through their production of SCFAs. SCFAs are major bacterial fermentation products, including mainly acetate, propionate, and butyrate. Propionate is mostly metabolized in liver while butyrate is the major energy source of colonocytes. The beneficial roles of SCFAs on the host have been demonstrated in IBD patients whose fecal SCFAs level are inversely correlated with the clinical symptoms [205].

The mechanisms by which SCFAs interact with host cells involve both intracellular and extracellular signaling pathways. Absorption of luminal propionate and butyrate by colonic epithelial cells inhibits the activity of histone deacetylases, which in turn promotes the hyperacetylation of histone and activates the targeted gene expression [206]. Butyrate and

propionate increase the expression of FOXP3, thereby induce the differentiation of T_{Reg} cells that suppress the inflammatory response [207]. Extracellular SCFAs interact with G protein-coupled receptors such as GPR41 and GPR43, which are expressed on colonic epithelial cells and immune cells, and mediate the activation of T_{Reg} cells and the production of IL-10 and TGF-β [208-209]. Overall, the immunosuppressive effects of probiotics and their metabolic products have been evaluated for treatment of IBD. Prebiotics that selectively increase beneficial bacteria and SCFAs production also provide a new perspective in IBD therapy.

Table 6. Protective roles of the gut microbiota in the IBD (Adapted from [158, 186]).

Bacteria species	Host type	Disease model	Mechanism of disease suppression
Bacteroides fragilis	Mouse	TNBS colitis	Induction of T_{reg} and IL-10 via PSA
Bacteroides vulgatus	*Il2*$^{-/-}$ Mouse	Spontaneous colitis	Suppression of inflammation
Bifidobacteria lactis	Rat	TNBS-colitis	Decreased levels of TNF and iNOS
Bifidobacteria infantis	Mouse	*Salmonella*-induced Enteritis	Induction of T_{reg}
Clostridium butyricum	*Rag2*$^{-/-}$ Mouse	DSS-colitis	Induction of T_{reg} and IL-10
Clostridial IV and XIVa	Mouse	DSS-colitis	Induction of T_{reg}
E. coli Nissle 1917	Mouse Human	DSS-colitis Ulcerative colitis	Decreased TLR2 and TLR4 activation
Faecalibacterium prausnitzii	Mouse Human	TNBS-colitis Ulcerative colitis	Induction of IL-10, Decreased IL-8 Induction of IL-10, Butyrate
Lactobacillus casei	Rat	TNBS-colitis	Decreased levels of cyclooxygenase2
Lactobacillus reuteri	*Il10* $^{-/-}$ Mouse	Spontaneous colitis	Decreased levels of TNF and IL-8

DSS, dextan-sulfate sodium ; TNBS, 2,4,6-trinitrobenzene sulphonic acid; Il2, interleukin 2; Il10, interleukin 10; *Rag2*, recombination-activating gene2; PSA, polysaccharide A; TNF, tumor necrosis factor; iNOS, inducible nitric oxide synthase, T_{reg}, regulatory T cells;

4. Glycosylation and immune system

Glycosylation is a key posttranslational modifications of proteins and lipids. Glycosylation occurs mainly in the lumen of the endoplasmic reticulum and Golgi apparatus [210]. Glycans are the fundamental macromolecules of the cells together with nucleic acids, proteins, and lipids. In contrast to the linear macromolecules (DNA and proteins), glycans display complex branched structures, and no template controls their biosynthesis. The diversity of glycans on glycoconjugates is derived from a stepwise combination of glycosyltransferases and glycosidases in response to genetic and environmental stimuli. Glycosylation contributes to a variety of biological functions including regulation of protein folding and secretion, structural stability, cellular development, receptor binding and activation [211]. Moreover, the structural diversity and unique signature of glycans among organisms allow cells to distinguish them from non-self components, therefore contribute to the immune tolerance and activation.

4.1 Sialylation

Sialic acids are nine-carbon keto-containing monosaccharides, widely expressed as terminal glycans on glycoconjugates of eukaryotic and some prokaryotic cells. The sialic acid family consists of over 60 naturally occurring monosaccharides which are derived from the core structure *N*-acetylneuraminic acid (Neu5Ac). The common modification of sialic acid is found at C-5 position and is replaced by a *N*-glycolyl group (Neu5Gc), a hydroxyl group (2-keto-3-deoxynonoic acid, KDN), and less commonly a 5-amino group (Neu). Additional modifications include substitution of the hydroxyl group on C-4, C-7, C-8, and C-9 positions to acetyl-, methyl-, sulfate-, lactyl- and phosphate groups all contribute to the diversity of sialic acid [212] **(Figure 9)**.

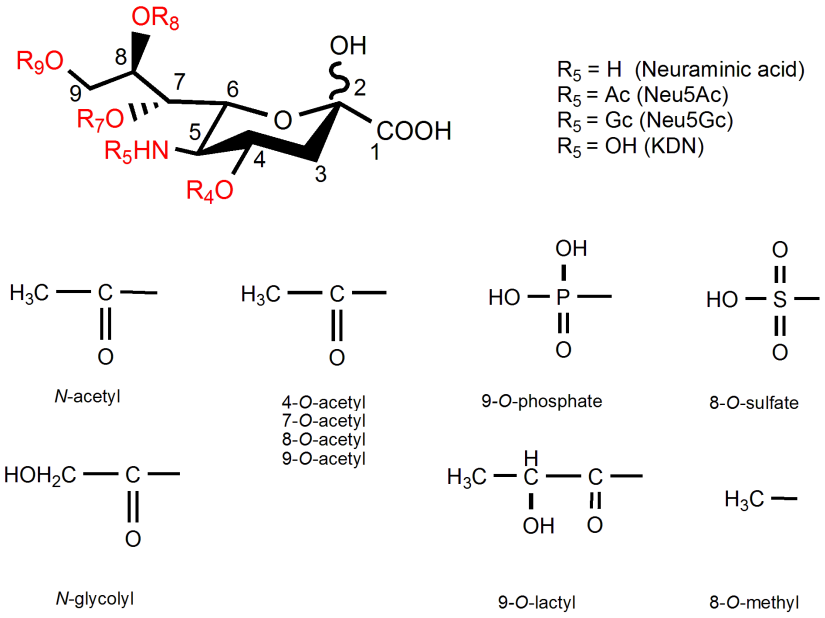


Figure 9. The diversity of sialic acids (Adapted from [212]). The diversity of sialic acids arises from a variety of modifications, The common modifications are identified at C-5 position, such as *N*-acetyl group (Neu5Ac), *N*-glycolyl group (Neu5Gc) or hydroxyl group (KDN), and less commonly 5-amino group (Neu). The hydroxyl group at C-4, C-7, C-8, and C-9 positions can be also modified by O-methyl, O-acetyl, O-lactyl, O-sulfate and O-phosphate group.

Sialylation is critical for a variety of cellular functions. The biosynthesis of sialic acid in the cytosol is catalyzed by a bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase (*GNE*) which converts UDP-GlcNAc into ManNAc-6-phosphate. Mutation on *Gne* is embryonic lethal in mice, suggesting the essential role of sialylation during development [213]. ManNAc-6-phosphate is further converted to Neu5Ac and transported into the nucleus where CMP-Neu5Ac synthase (*CMAS*) formed CMP-Neu5Ac. The nucleotide activated sugar, CMP-Neu5Ac, is then imported into the Golgi compartment, where the sialylation of glycoconjugates is catalyzed by a group of 20 sialyltransferases. The metabolism of sialic acid and sialoconjugates is summarized in **Figure 10**.

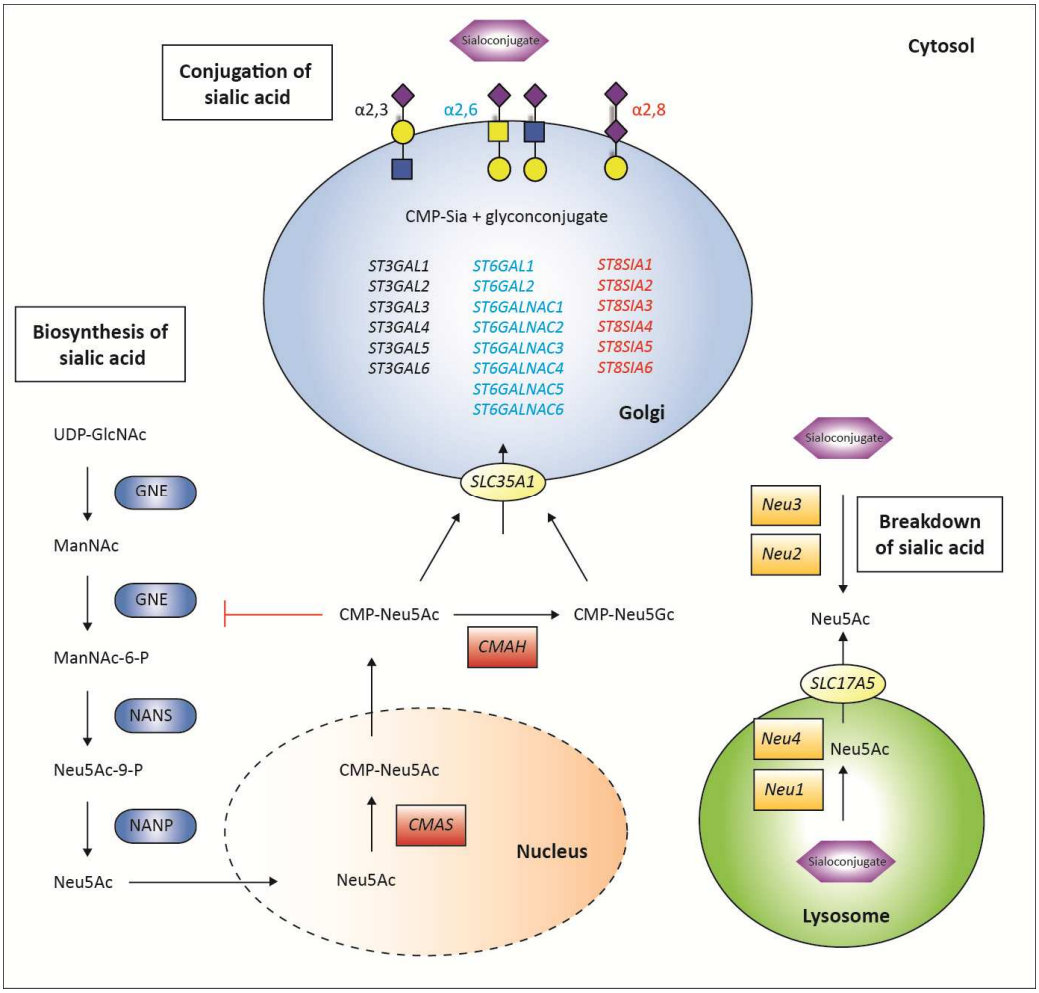


Figure 10. Metabolism of sialic acid (Adapted from [214]). The biosynthesis of sialic acid begins with the conversion of UDP-GlcNAc to *N*-acetylmannosamine (ManNAc) followed by phosphorylation to ManNAc-6-phosphate by the bifunctional enzyme UDP-GlcNAc-2-epimerase/ManNAc kinase (*Gne*). ManNAc-6-phosphate is then converted to Neu5Ac-9-phosphate and followed by dephosphorylation into Neu5Ac. Neu5Ac is activated by CMP-Neu5Ac synthase (*CMAS*) to form CMP-Neu5Ac in the nucleus. CMP-Neu5Ac is then imported via a specific transporter (*SLC35A1*) into the lumen of the Golgi apparatus where the synthesis of sialoconjugates is catalyzed by a variety of sialyltransferases. The breakdown of sialoconjugates is mediated by sialidases (*Neu1, Neu4*) in lysosome or (*Neu2* and *Neu3*) in the cytosol.

To date, twenty sialyltransferases are identified in mammals and characterized into four families (*i.e. St3gal*, *St6gal*, *St6galnac*, and *St8Sia*) based on their linkage and substrate specificity. The known substrates and the biological roles of sialyltransferases are summarized in **Table 7**.

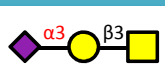
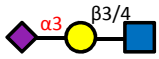
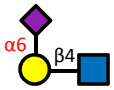
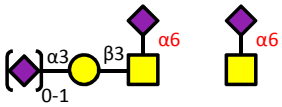
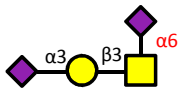
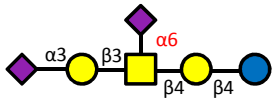
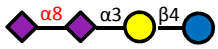
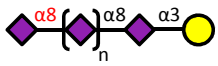
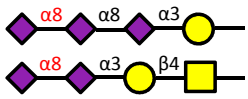
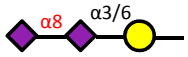
4.2 Sialylation and sialyltransferases

The *St3gal* family contains six sialyltransferases (*St3gal1- St3gal6)* which transfer sialic acid in α2,3 linkage to Gal. *St3gal1* regulates $CD8^+$ T cell apoptosis and promotes the formation of $CD8^+$ T cell memory [215]. *St3gal3* mediates eosinophil apoptosis via synthesis of siglec-F ligand, as evidenced by an increasing allergic eosinophilic response in *St3gal3* deficient mice [216]. *St3gal4* and *St3gal6* are both involved in the synthesis of the selectin ligand sialyl Lewis X (sLe^x), which plays a critical role in leukocyte trafficking, as evidenced by impaired neutrophil recruitment and lymphocyte homing in double-deficient mice [217-218]. Moreover, *St3gal4* is also responsible for synthesizing milk α2,3-sialyllactose, which affects the colonization of gut microbiota and the susceptibility of mice to experimental colitis [219]. *St3gal5* is mainly expressed in the neural tissues and catalyzes the first step of ganglioside GM3 synthesis. Mutation in *St3gal5* leads to a neurological disorder characterized by recurrent seizures and intellectual disability [220].

Two sialyltransferases *St6gal1-2* and six sialyltransferases *St6galnac1-6* transfer sialic acid in α2,6 linkage on Gal and GalNAc acceptors, respectively. *St6gal1* mediates the synthesis of the siglec-2 ligand (CD22), which modulates the B cell receptor activation [221], while *St6gal2* expression is restricted to the brain, suggesting its potential function in neuronal development [222]. The biological functions of the *St6galnac* family are less understood. Most of these sialyltransferases mediate the sialylation on O-GalNAc glycans, and glycolipids, suggesting their potential roles in regulating cellular adhesion and signaling [223].

Finally, the *St8Sia* family contains six sialyltransferases (*St8Sia1-6*), which elongate sialic acid in α2,8 linkage and form polysialic acid chains. In mammals, the presence of polysialic acid is restricted to a small number of glycoproteins and gangliosides [223]. The best-characterized poly-sialylated glycoprotein is neural cell adhesion molecule (NCAM). NCAM mediates adhesive property and neural plasticity of cells through the intermolecular repulsion generated from negatively charged-polysialic acid chains [224]. Recently, *St8Sia2* and *St8Sia4* have also been associated with the polysialylation on the synaptic cell adhesion molecule (SynCAM1) in the brain [225].

Table 7. Summary of human sialyltransferase family (Adapted from [223, 226]).

Sialyltransferases	Synthesized products	Biological functions
St3gal1 St3gal2		CD8⁺ T cell homeostasis (CD43 sialylation)
St3gal3		Brain development (Ganglioside) Allergic inflammation (Siglec-F ligand)
St3gal4 St3gal6		Lymphocyte trafficking (Selectin ligand)
St3gal5		GM3 synthase
St6gal1 St6gal2		B cell receptor activation , regulation of IgG activity
St6galnac1 St6galnac2		Synthesis of STn antigen
St6galnac3 St6galnac4		ST6GalNAc3 prefer on Glycolipid ST6GalNAc4 prefer on O-Glycans
St6galnac5 St6galnac6		GD1α synthase
St8Sia1		Cholesterol and ganglioside metabolism; GD3 synthase
St8Sia2 St8Sia3		Synaptic plasticity (Polysialic acid on NCAM)
St8Sia4		Neuronal and glial differentiation (NCAM) Polysialic acid on Neuropilin-2
St8Sia5		Neuronal cell development Synthesis of GD1c, GT1a, GT3,
St8Sia6		Synthesis sialylated O-glycans

4.3 Sialylation and microorganisms

Mucosal surface is covered with a thick layer of mucus that contains a multitude of glycan epitopes. Given the abundance of sialic acids as the outermost carbohydrates on cell membranes, numerous bacteria and viruses utilize host-derived sialylated glycans as receptors for adhesion and infection. Recently, several sialic acid-binding agglutinins,

adhesins, and lectins have been identified in microbes, and many are considered as virulence factors for infection. For instance, *Vibrio cholera*e, *H. pylori*, *E. coli* enterotoxin, and botulinum toxin target specific mucosal sialylated glycans [227-229]. Moreover, bacteria such as commensal Bifidobacteria and Bacteroides spp. secrete sialidases and hydrolyze host-derived sialylated glycans for nutritional purposes. Besides using sialidases for nutrient acquisition, several pathogens also exploit sialidases to unmask underlying host ligands for further adherence and infection. For instance, *C. perfringens* and *V. cholera* sialidases are critical virulence factors, which reveal underlying cryptic ligands as binding sites for epsilon toxin and cholera toxin, respectively [230-231]. The sialidase-producing bacteria and substrate specificity are summarized in **Table 8**.

Table 8. Sialidase-producing bacteria and substrate specificity (Adapted from [232-233]).

Mucosal site	Sialidase-producing bacteria	Substrate
Gastrointestinal tract	*Bacteroides fragilis*	3SL, 6SL, LST, GM1, fetuin, mucin
	Bacteroides thetaiotaomicron	HMO sialyllactose, mucin
	Bacteroides vulgatus	HMO sialyllactose, mucin
	Bifdobacterium longum	HMO sialyllactose
	Bifdobacterium infantis	HMO sialyllactose
	Clostridium perfringens	3SL, 6SL, GM1, mucin
	Salmonella typhimurium	3SL, 6SL, colominic acid, fetuin, mucin
	Vibrio cholerae	Ganglioside GM1, GM2, GM3, GD1
Buccal cavity	*Porphrymonas gingivalis*	Sialyllactose, α1-acid glycoprotein
	Tannerella forsythia	3SL, 6SL, colominic acid, fetuin, mucin
Respiratory tract	*Haemophilus influenzae*	Uncharacterized sialic acid scavenge
	Streptococcus pneumoniae	3SL, 6SL, IgA1, lactoferrin,
	Pasteurella multocida	3SL, 6SL, GM1, GD1a, colominic acid
	Pseudomonas aeruginosa	Synthetic MU-Neu5Ac
Reproductive tract	Consortia in bacterial vaginosis	IgA, mucins
	Gardnerella vaginalis	Synthetic MU-α-Neu5Ac

LST, Sialyl-lacto-N-tetrarose; MU-Neu5Ac, 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid

Notably, bacteria such as *E. coli* do not express sialidase genes but are capable of scavenging free sialic acid as an energy source from the environment. Other bacteria such as *B. thetaiotaomicron* express sialidases but do not encode a sialic acid lyase gene (NanA), which is required for the initial step of sialic acid catabolism. This finding suggests that *B. thetaiotaomicron* may utilize the sialidase activity to unmask and access the underlying glycan structures. Such a Bacteroides-derived sialidase activity has recently been demonstrated to promote the proliferation of the enteropathogens *Salmonella typhimurium* and *Clostridium difficile* [234].

In addition to their nutritional contributions, sialic acids can be incorporated into microbial surface glycans, and thereby mediate the formation of self-associated molecular patterns (SAMPs). Recognition of SAMPs by host serum protein factor H and members of the siglec family dampens the activation of the complement system and inhibits innate immune responses, and thereby avoids pathogens being attacked by the host immune system. The best-known examples are pathogens such as *E. coli* K1, and *Neisseria meningitidis.* They can *de novo* synthesize polysialic acids and express capsular polysaccharides to avoid immune attack [235-236], as evidenced by the reduction of bacteremia and systemic infection in rat through the selective degradation of *E. coli* K1 capsular polysialic acids [237]. In addition, bacteria such as *Haemophilus influenzae*, lack *de novo* synthesis enzymes but can scavenge exogenous sialic acid and activate it into CMP-sialic acid for subsequent sialylation of surface lipooligosaccharide [238]. Interestingly, several pathogens have evolved various mechanisms to acquire sialic acid either by scavenging or *de novo* synthesis (**Figure 11**).

Overall, sialylation has a profound impact on regulating the host immune system and microbial recognition. Therefore, regulation on microbial sialic acid metabolism and surface sialoconjugates can influence the interplay between the host cells and microorganisms.

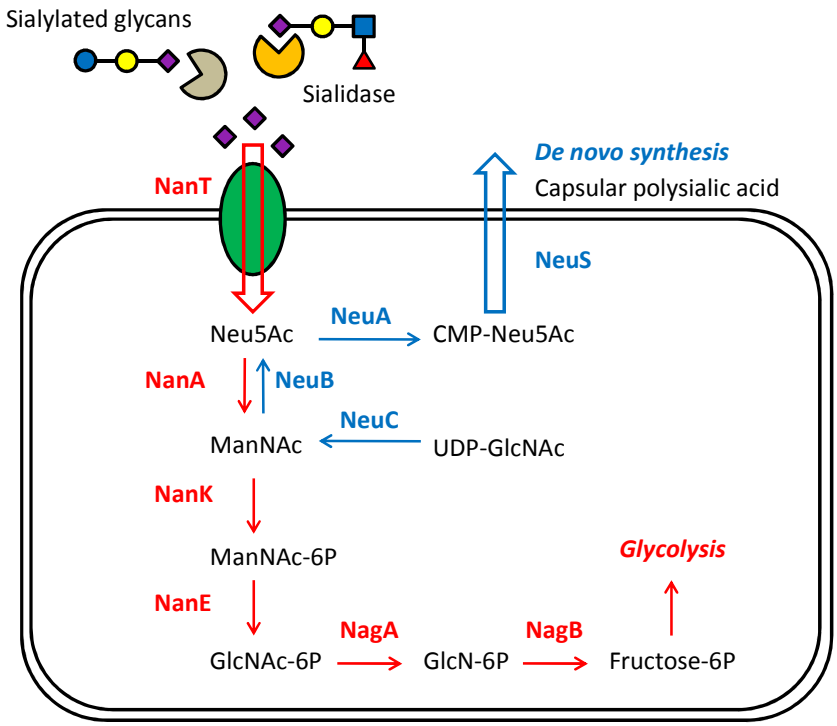


Figure 11. *De novo* synthesis and metabolism of sialic acid in bacteria (Adapted from [239]). Summary of sialic acid catabolism (red) and de novo synthesis (blue) pathway. De novo synthesis of sialic acid begins with the conversion of UDP-GlcNAc to ManNAc by UDP-GlcNAc epimerase (NeuC), followed by condensation of ManNAc to Neu5Ac by sialic acid synthase (NeuB). Finally, CMP-sialic acid synthase (NeuA) catalyzes formation of CMP-Neu5Ac, providing an activated donor substrate for sialylation. NeuA: CMP-sialic acid synthase; NeuB: sialic acid synthase; NeuC, UDP-GlcNAc epimerase NanT: sialic acid permease; NanA: *N*-aceylneuraminic lyase; NanK: *N*-acetylmannosamine kinase; NanE: *N*-acetylmannosamine-6P epimerase; NagA: *N*-acetylglucosamine-6-P deacetylase; NagB: Glucosamine-6-P deaminase.

Aim of the study

Glycans derived from maternal milk, diet, and mucosa greatly influence the composition of intestinal microbiota and mediate gastrointestinal physiology. However, the direct contributions of glycans on regulating intestinal bacteria remain largely unclear. Imbalance in gut microbiota is frequently observed in response to environmental stress and in various disease conditions. Despite such well documented alterations, the mechanisms and consequences of dysbiosis on disease development remain elusive. Knowledge of specific glycans functions may be useful to develop prebiotics and targeted therapies against intestinal inflammation manifested by microbial dysbiosis. The aim of this study is to characterize the functional roles of glycans, especially sialic acid, in regulating intestinal bacteria and to determine their contributions to mucosal immunity.

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RESULTS : Manuscript 1

Sialic acid catabolism drives intestinal inflammation and microbial dysbiosis in mice

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Yen-Lin Huang[1], Christophe Chassard[2‡], Martin Hausmann[3], Mark von Itzstein[4], Thierry Hennet[1*]

[1] Institute of Physiology and Zurich Center of Integrative Human Physiology, University of Zurich, Zurich, Switzerland.

[2] Laboratory of Food Biotechnology, Institute of Food, Nutrition and Health, ETH Zurich, Zurich, Switzerland.

[3] Division of Gastroenterology and Hepatology, University Hospital of Zurich, Zurich, Switzerland.

[4] Institute for Glycomics, Griffith University, Gold Coast Campus , Gold Coast, Queensland 4222, Australia

* Corresponding author: Thierry Hennet, Institute of Physiology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

Email : thennet@access.uzh.ch

Tel : +41 44 635 5080

Fax : +41 44 635 6814

‡ Present address: Institut National de la Recherche Agronomique, UR 545 URF, 15000 Aurillac, France

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Abstract

Rapid shifts in microbial composition frequently occur during intestinal inflammation, but the mechanisms underlying such changes remain elusive. We here demonstrate that an increased caecal sialidase activity is critical in conferring a growth advantage for *Escherichia coli* during intestinal inflammation in mice. This sialidase activity originated from *Bacteroides vulgatus*, whose intestinal levels expanded after dextran-sulfate sodium administration. Increased sialidase activity mediates the release of sialic acid from intestinal tissue, which promotes the outgrowth of *E. coli* during inflammation. The outburst of *E. coli* exacerbated the inflammatory response by stimulating the production of pro-inflammatory cytokines by intestinal dendritic cells. Oral administration of a sialidase inhibitor and low levels of intestinal α2,3-linked sialic acid decreases *E. coli* outgrowth and the severity of colitis in mice. Regulation on sialic acid catabolism opens new perspectives for the treatment of intestinal inflammation as manifested by *E. coli* dysbiosis.

Introduction

The intestinal microbiota has emerged as a key player in the regulation of physiological pathways and in the development of diseases. Along with intestinal diseases, such as necrotizing enterocolitis[1] and inflammatory bowel disease[2], gut microbiota contribute among others to the etiology of diabetes[3], asthma[4], autoimmunity[5], and cancer[6]. Accordingly, much effort has been dedicated to understanding the factors influencing the composition of the intestinal microbiota to maintain or restore health in the host organism.

Carbohydrates are a major class of food products that profoundly affect the gut microbiota. Whereas most monosaccharides are absorbed by the small intestine, oligo- and polysaccharides are not digested in the upper gastrointestinal tract and reach the colon intact. The impact of complex carbohydrates on microbial composition is based on the expression of specific hydrolases[7], which enable some bacterial species to process and utilize breakdown products as nutrients, thereby conferring a proliferative advantage over bacteria that cannot process complex carbohydrates[8]. The first carbohydrates ingested just after birth are provided by breast milk, which is a rich source of lactose and oligosaccharides[9]. The uptake of milk oligosaccharides coincides with the microbial colonization of the gut and favors the proliferation of bacteria equipped with carbohydrate processing enzymes, such as *Bifidobacterium* and *Bacteroides spp* that are enriched in breast-fed infants[10].

In addition to food carbohydrates, several intestinal bacteria can process host-derived carbohydrates, which are prominent constituents of mucosal layers. Besides providing carbon sources for bacterial growth, released host-derived carbohydrates influence gene expression in the microbiota, thereby affecting the virulence of pathogenic bacteria as demonstrated by the regulation of virulence factors in enterohaemorrhagic *E. coli* by fucose[11]. Other host-derived carbohydrates, such as sialic acids, are taken up by bacteria lacking *de novo* biosynthetic pathways for these sugars, and incorporated into bacterial capsule and lipooligosaccharides[12]. The decoration of bacterial glycoconjugates with sialic acid protects microbes from recognition by the host immune system[13] and regulates the host immune response through interactions with sialic acid-binding lectins[14]. Finally, the interplay between

intestinal microbiota and host glycosylation is not limited to the utilization of host glycans by bacteria. Sialic acids as terminal residues on intestinal glycoconjugates are a prime target for bacterial adhesins and toxins from *Vibiro cholerae*, *Helicobacter pylori* and *E. coli* [15-16].

The structural complexity of carbohydrates, either ingested in the form of milk oligosaccharides, or expressed as host-derived glycans, hampers the elucidation of their impact on the gut microbiota. Accordingly, little is known about the relevance of specific carbohydrates on microbiota composition and on intestinal physiology. The application of mice deficient for glycosyltransferases enables the investigation of interactions between defined carbohydrates, intestinal microbes, and the host immunity. For example, a study of α1,2 fucosyltransferase *Fut2* knockout mice has recently demonstrated the interplay between fucosylated glycans and diet polysaccharides on shaping the gut microbiota[17]. The study of α2,3 sialyltransferase *St3gal4* knockout mice (ST), which mediates α2,3-sialyllactose (3SL) synthesis in mammary gland, has established the role of the milk oligosaccharide on the gut microbiota and thereby on the susceptibility of mice in dextran sulfate sodium (DSS)-induced acute[18] and chronic colitis[19]. Through the investigation of DSS-mediated colitis in ST mice and the modulation of the intestinal microbiota by selective antibiotic treatment, the present study reveals the critical role of α2,3-linked sialic acid in establishing a niche for intestinal *E. coli* after lactation and during intestinal inflammation.

Results

Gut microbiota change during DSS induced colitis

To unravel the relationship between α2,3-linked sialic acid and the intestinal microbiota, and to identify the mechanisms of α2,3-linked sialic acid effects on colitis development, we have first addressed the impact of intestinal bacterial groups on colitis by treating mice with a panel of antibiotics. Correlations between the resulting changes in microbial composition and susceptibility to DSS-mediated colitis pointed to specific bacterial families possibly regulating the severity of colitis in wild type (WT) and ST mice. In fact, the composition of colonic bacteria in WT and ST mice differed at the adult stage. The most abundant bacterial family in WT mice was *Ruminococcaceae*, reaching 44% of total bacteria. By contrast, *Ruminococcaceae* only represented 10% of colonic bacteria in ST mice, whereas *Porphyromonadaceae* dominated by reaching 37% (**Fig. 1a**). The bacterial composition of mice undergoing intestinal inflammation induced by DSS changed dramatically, as seen by a strong expansion of *Bacteroidaceae* and *Enterobacteriaceae* in WT mice. ST mice, which were less susceptible to DSS than WT mice , also showed increased *Bacteroidaceae* levels during DSS challenge, whereas *Enterobacteriaceae* remained at low level. Sequence analysis at the genus level indicated that the *Escherichia* and *Shigella* accounted for the observed increase of *Enterobacteriaceae* in WT mice, and *Bacteroides* accounted for the increase of *Bacteroidaceae* in both WT and ST mice under DSS challenge (**Fig. 1b**).

Antibiotics effect on DSS induced colitis

To determine whether a specific group of bacteria accounted for the different response to DSS, we treated WT mice with a panel of antibiotics before DSS challenge. Vancomycin, neomycin and penicillin exacerbated the severity of DSS-induced colitis as monitored by loss of body weight (**Fig. 2a**). Streptomycin was the only antibiotic that attenuated the loss of body weight during colitis, whereas chloramphenicol and metronidazole did not have much impact on the course of the inflammatory response (**Fig. 2a**). To exclude any damaging effect caused by the use of antibiotics on intestinal barrier function, we assessed epithelial permeability by measuring the leakage of orally administered FITC-dextran into the

bloodstream. Permeability was only significantly increased after DSS ingestion, but not after antibiotic treatment (**Fig. 2b**). We also tested the effect of a short-term treatment with antibiotics to exclude possible adaptations of the host mucosa to three weeks of altered microbiota composition. Focusing on vancomycin (**Fig. 2c**) and streptomycin (**Fig. 2d**), we could reproduce the worsening and improving effects of these two antibiotics by only administering vancomycin and streptomycin during DSS challenge. The protective effect of streptomycin towards DSS challenge was even more pronounced in a short term treatment compared with a three week pre-treatment in both WT and ST mice. The extent of intestinal inflammation was confirmed by measuring the length of the colon in treated mice. The shortening of colon length induced by DSS ingestion was aggravated by vancomycin and reduced by streptomycin (**Fig. 2e**). The impact of vancomycin and streptomycin treatment on the intestinal microbiota of WT mice was analyzed by 16S rRNA pyrosequencing and compared to the changes observed during DSS challenge. Vancomycin induced a strong increase of *Enterobacteriaceae*, which raised to 27% of total bacteria, whereas *Enterobacteriaceae* remained below 0.1% of total bacteria in streptomycin-treated mice (**Fig. 2f**). Under both antibiotics, *Bacteroidaceae* expanded to represent the major bacterial family, but the increase in *Bacteroidaceae* did not directly correlate with the severity of DSS-induced colitis. In contrast, the abundance of *Enterobacteriaceae* correlated with the magnitude of colitis. Overgrowth of *Enterobacteriaceae* and several *Bacteroidaceae* spp. during intestinal inflammation is well documented, although the mechanisms underlying such expansions have not been identified in previous studies[20-21].

Expansion of *E. coli* during DSS induced colitis

To verify which species of *Enterobacteriaceae* expanded during intestinal inflammation, we applied specific primers targeting the β-glucuronidase *uidA* gene[22] and confirmed a significant increase of *E. coli* in DSS-challenged mice and in vancomycin-treated mice (**Fig. 3a**). In contrast, a significant reduction of *E. coli* was observed in both WT and ST mice treated with streptomycin. Overall, *E. coli* levels correlated with the severity of colitis in all genotypes and antibiotic treatments tested. Of note, the level of *E. coli* in adult ST mice was 2 orders of

magnitude lower than in WT mice, indicating that decreased exposure to α2,3-linked sialic acid, because of reduced sialylation of host glycans and the absence of 3SL in milk ingested during lactation, was accompanied by low level of intestinal *E. coli*. The importance of milk 3SL during lactation at promoting the low-level colonization of *E. coli* was also visible in WT mice that were fostered by ST mother during lactation. WT mice fed on 3SL-deficient milk (WTXF) showed lower *E. coli* levels than littermates fed on normal milk. Similarly, ST mice fed on normal milk showed elevated *E. coli* levels compared with littermates fed on 3SL-deficient milk (**Fig. 3b**). The relative abundance of intestinal *E. coli* in cross-fostered mice also reflected the severity of DSS-induced colitis[18]. Overall, these data confirmed that neonatal exposure to milk 3SL contributed to the bacterial colonization of the intestine, especially by *E. coli*, and pointed to a role of *E. coli* in affecting the severity of colitis induced by DSS.

Exposure to sialic acid promotes *E. coli* expansion

To address the impact of 3SL on *E. coli* proliferation, we isolated various strains of commensal *E. coli* from the colon of WT mice during DSS-induced colitis. The identity of the isolated bacteria with *E. coli* was confirmed by sequencing universal stress protein *uspA* gene[23], gyrase *gyrB* gene[24] and by biochemical testing using the API20E *Enterobacteriaceae* detection system. The culture of the isolated *E. coli* strain EHV2 in minimal medium containing unique monosaccharides as carbon source confirmed that *N*-acetylneuraminic acid was a preferential sources of energy for *E. coli* (**Fig. 4a**). In contrast to free *N*-acetylneuraminic acid, the milk oligosaccharides 3SL and α2,6-sialyllactose (6SL) did not support *E. coli* growth *in vitro* (**Fig. 4b**). Proliferation could however be restored by adding sterile-filtered caecal fluid from WT mice to the culture medium, whereas *E. coli* growth was more robust in 3SL than in 6SL containing minimal medium. Because *E. coli* do not produce sialidases, the restoration of bacterial growth pointed to the presence of an α2,3-preferential

sialidase activity in caecal fluid. Such a sialidase activity was confirmed in caecal fluid and shown to increase significantly during DSS-induced colitis in both WT and ST mice (**Fig. 4c**). The substrate specificity of this caecal fluid sialidase was demonstrated by HPLC analysis after incubation of 3SL with caecal fluid (**Supplementary Fig. 1**). The sialidase activity was also increased in the caecal fluid of WT mice treated with streptomycin or vancomycin. By contrast, the sialidase activity was strongly decreased in WT mice treated with a broad spectrum antibiotic cocktail consisting of ampicillin, vancomycin, metronidazole, and neomycin, which supported the bacterial origin of the sialidase activity in caecal fluid (**Fig. 4d**). To identify the source of this sialidase activity, we focused on bacterial groups, such as commensal *Bacteroides* and *Bifidobacteria* species that are known to secrete sialidases[25]. Host derived sialidases were unlikely candidates since vertebrate sialidases are unstable as soluble proteins in the extracellular space[26].

Bacteroides vulgatus sialidase releases sialic acid

As *Bacteroides* species were strongly increased in the gut of mice challenged with DSS (**Fig. 1b)** as well as in mice treated with streptomycin or vancomycin (**Fig. 2f**), we searched for sialidase genes in the caecum of DSS-challenged mice using a series of PCR primers encompassing known *Bacteroides* sialidase sequences in the glycoside hydrolase family 33 of the Cazy database. This analysis revealed a 100-fold increase in copy number of the *Bacteroides vulgatus* BVU_4143 sialidase gene (Gene ID: 5305102) in WT mice treated with DSS, whereas no change was detected in ST mice **(Fig. 4e)**. The abundance of *B. vulgatus* also increased accordingly in the colon of WT mice during DSS-induced colitis (**Supplementary Fig. 2**). Moreover, the abundance of the BVU_4143 sialidase gene increased in WT mice treated with streptomycin and vancomycin, but decreased in mice treated with antibiotic cocktail **(Fig. 4f)**. The sialidase activity of BVU_4143 was confirmed after expression as a recombinant protein by demonstrating its ability to hydrolyze the aryl substrate 4-MU-NeuNAc (**Supplementary Fig. 3a**) and pNP-NeuNAc (**Supplementary Fig. 3b**). The sialidase activity of

recombinant BVU_4143 was inhibited by the sialidase inhibitor *N*-acetyl-2,3-didehydro-2-deoxyneuraminic acid (Neu5Ac2en) and lost by heat treatment. The addition of recombinant BVU_4143 to minimal media containing 3SL or 6SL enabled the growth of *E. coli* EHV2 on these oligosaccharides (**Supplementary Fig. 3c**), as observed for the restoration of *E. coli* EHV2 proliferation by addition of sterile-filtered caecal fluid to culture medium containing 3SL and 6SL **(Fig. 4b)**. Overall, these results indicate that the expansion of *B. vulgatus* and the concomitant increased sialidase activity during DSS-induced colitis enables the sialic acid-dependent outgrowth of *E. coli* during inflammation.

Sialic acid is required for sustaining *E. coli* colonization

The dependence of *E. coli* on sialic acid *in vivo* was investigated by deleting the sialic acid transporter *nanT* gene[27]. Disruption of *nanT*, the first committed step in the sialic acid utilization pathway, abolished growth of the mutant *E. coli* strain in a sialic acid-containing minimal medium, but not growth in glucose containing medium (**Fig. 5a**). By contrast, the disruption of mannose transporter *ManX* did not affect the growth of *E. coli* in both glucose and sialic acid-containing medium. To investigate the in vivo colonization efficiency, *nanT* mutant and parental *E. coli* were gavaged at equal amounts of each 10^6 cells to ampicillin-pretreated mice. Colonization efficiency was determined over a period of 10 days after inoculation by counting *E. coli* isolated from freshly isolated feces samples. Both strains were colonized at 10^8-10^9 colony-forming units per gram feces in WT mice by two days after inoculation. Parental *E. coli* remained stable over 10 days, but *nanT* mutants decreased dramatically over the same period (**Fig. 5b**). The same experiment performed in ST mice showed that even parental *E. coli* did not maintain their original levels in an environment deficient of α2,3-linked sialic acid (**Fig. 5c**). The comparison of competitive index between parental and *nanT E. coli* in the intestines of WT and ST mice suggested that the growth advantage of parental *E. coli* was associated with the local availability of sialic acid (**Fig. 5d**). The levels of free Neu5Ac in the caecal fluid were indeed higher in WT mice than in ST mice (**Fig. 5e**), thereby correlating with the occurrence of intestinal *E. coli* in WT and ST mice (**Fig. 3a**). The concentrations of Neu5Ac measured in the caecum of WT mice treated with

streptomycin and vancomycin (**Fig. 5f**) also matched the abundance of *E. coli* in these mice (**Fig. 3a**). In the late-stage of DSS-induced colitis, the levels of free Neu5Ac decreased in WT mice and relatively increased in ST mice, which reflected increased sialic acid usage by *E. coli* and increased sialidase activity (**Fig. 4c**) during intestinal inflammation. Accordingly, these results were consistent with the hypothesis that *E. coli* outgrowth in the intestine depends on the release of sialic acid from host glycans.

Sialidase inhibition lowers *E. coli* expansion and colitis

Based on the requirement for sialidase activity to cleave α2,3-linked sialic acid and to promote *E. coli* proliferation during intestinal inflammation, we hypothesized that sialidase inhibition would decrease both the expansion of *E. coli* during DSS-induced colitis and the severity of the inflammatory response. We first confirmed the effectiveness of the sialidase inhibitor Neu5Ac2en at preventing *E. coli* growth in presence of 3SL and caecal fluid *in vitro* (**Fig. 6a**). Next, we confirmed the effectiveness of Neu5Ac2en at reducing the release of sialic acid *in vivo* by showing decreased levels of free Neu5Ac in the caecum of WT mice treated with the sialidase inhibitor **(Fig. 6b).** Oral administration of Neu5Ac2en to WT mice during DSS challenge also prevented the outgrowth of *E. coli* during inflammation, as seen by a decrease of *E. coli* levels by 2-3 orders of magnitude (**Fig. 6c**), and decreased the severity of DSS-induced colitis as assessed by change in body weight (**Fig. 6d**) and colon length (**Fig. 6e**). Neu5Ac2en treatment also reduced the loss of colonic architecture and leukocyte infiltration (**Fig. 6f**), although without reaching statistical significance (**Fig. 6g**). Neu5Ac2en treatment was by contrast ineffective in ST mice challenged with DSS (**Supplementary Fig. 4**), which was expected considering the low levels of sialidase-producing *Bacteroides* spp. and low sialidase activity in the caecum of ST mice. Overall, these data demonstrated that inhibition of caecal sialidase activity significantly reduced the outburst of *E. coli* and hence the severity of colitis in mice.

E. coli intensifies dendritic cell activation

The question as to how *E. coli* proliferation affected intestinal inflammation remained open. We therefore assessed the pro-inflammatory potential of *E. coli* on intestinal $CD11c^+$ dendritic cells (DCs). Previous work has shown increased $CD11c^+$ DC infiltration to the colonic mucosa of WT mice compared to ST mice, suggesting a critical role of DCs during intestinal inflammation[19]. We examined the stimulatory effect of *E. coli* EHV2 and of the *Bacteroides thetaiotaomicron* on mesenteric lymph node-derived $CD11c^+$ DCs. *B. thetaiotaomicron* was chosen as reference because *Bacteroides* represents a major group of intestinal bacteria, which expanded in both WT and ST mice during DSS-mediated colitis (**Fig. 1b**). Stimulation of $CD11c^+$ DCs with fixed *E. coli* increased the expression of the activation markers MHC-II, CD86, and CD40, whereas stimulation with fixed *B. thetaiotaomicron* failed to activate $CD11c^+$ DCs (**Fig. 7a**). The pro-inflammatory effect of *E. coli* was not limited to mouse DCs as stimulation of the human monocytic cell line THP-1 also increased the expression of the activation marker CD54 (**Fig. 7b**). The effect of *E. coli* was even more pronounced when measuring the secretion of pro-inflammatory cytokines from stimulated mouse $CD11c^+$ DCs. The levels of IL-6, TNF-α, and IL-12p40 produced after *E. coli* stimulation exceeded those reached after stimulation with LPS at 500 ng ml^{-1}. Under identical conditions, *B. thetaiotaomicron* did not increase cytokines production (**Fig. 7c**).

Overall, this study demonstrated that the expansion of commensal *E. coli* following the alteration of epithelial integrity caused by DSS uptake was mediated by increased exposure to sialic acid, and that overgrowth of *E. coli* exacerbated intestinal inflammation by stimulating the release of pro-inflammatory cytokines from intestinal DCs.

Discussion

Multiple studies have documented that intestinal inflammation is frequently accompanied by imbalanced microbiota. Such a dysbiosis is often characterized by a relative increase of facultative anaerobic *Enterobacteriaceae*[28]. Different factors such as nitrate[29] and enterobactin[30] promote *Enterobacteriaceae* expansion. The present study underlines the contribution of host glycosylation, specifically of α2,3-linked sialic acids, in enabling the proliferation of *Enterobacteriaceae* during intestinal inflammation in mice. Exposure to α2,3-linked sialic acids begins during lactation with the uptake of the milk oligosaccharide 3SL. After weaning, sialylated host glycans constitute the main source for the carbohydrate. Whereas *Enterobacteriaceae* genomes encode various glycosidases, bacteria such as *E. coli* cannot digest sialylated oligosaccharides. Therefore, their growth relies on scavenging free monosaccharides released by glycosidases of other bacteria. Our comparative study of monosaccharides showed that the sialic acid yielded the fastest growth of *E. coli* among the main monosaccharides encountered in mammalian glycans. This finding is consistent with previous work showing that sialic acid catabolism conferred a growth advantage to intestinal *E. coli* [31-32].

The growth advantage provided by sialic acid was dependent on a sialidase displaying a preference for α2,3-linked over α2,6-linked sialic acids, and which increased during intestinal inflammation. Commensal *E. coli* do not express sialidases to liberate host sialylated glycans, therefore the access to bound sialic acids depends on secreted sialidases, such as the BVU_4143 sialidase identified in our study. We also detected other sialidase genes in colitogenic mice, such as sequences sharing similarity with sialidase genes encoded by *Bacteroides fragilis* and *Parabacteroides distasonis*, although the abundance of these sialidase sequences did not vary between mouse genotypes and during DSS-mediated colitis. The dependence of *E. coli* on sialidases secreted by *Bacteroides spp.* may explain the parallel increased abundance of *Bacteroides spp*. and *E. coli* observed in patients with colitis[33]. A recent study also demonstrated that the commensal sialidase-producing *B. thetaiotaomicron* was associated with proliferation of *Salmonella enterica* typhimurium and *Clostridium difficile*[34]. By contrast, colonization of mice with a sialidase-deficient mutant reduced free sialic acid levels and thereby impaired the expansion of *C. difficile*.

The decreased severity of DSS-induced colitis in mice treated with sialidase inhibitor demonstrated the contribution of sialic acid in *E. coli* expansion and in the ensuing inflammatory response. Administration of free sialic acid to mice prior or during DSS challenge however failed to affect the levels of intestinal *E. coli* and the severity of colitis (**Supplementary Fig. 5**). Considering that monosaccharides are absorbed in the small intestine and only minute amounts reach the colon, oral supplementation with sialic acid is thus unlikely to influence the outgrowth of *E. coli* in the colon. Therefore, the release of sialic acid from host glycans is critical in promoting the growth advantage of *E. coli*. The increase in sialylation of intestinal mucins during colitis[35-36] likely facilitates the local release of free sialic acid during inflammation.

Our findings showed that commensal *E. coli* was a potent activator of a pro-inflammatory response in intestinal DCs. The activation of DCs was likely induced by surface lipopolysaccharides (LPS) triggering Toll-like receptor-4 signaling. The strong pyrogenic effect of commensal *E. coli* over the one induced by *B. thetaiotaomicron* matches previous findings showing that *E. coli* LPS was more active than *Bacteroides spp*-derived LPS at inducing TNFα production[37]. Similar differences in pyrogenicity were also noted in mouse models[38-39]. These results demonstrate that increased *E. coli* levels likely exacerbate inflammation through activation of mucosal immune cells such as DCs. Several intestinal bacteria regulate mucosal immunity, thereby affecting the occurrence of Th17 cells in the lamina propria in the case of segmented filamentous bacteria[40-41], or the induction of $Foxp3^+$ Treg cells in the case of a group of *Clostridium spp*[42] and *Bacteroides fragilis*[43]. We cannot exclude that the disappearance of inflammation-lessening bacteria also affects the severity of colitis alongside the expansion of pro-inflammatory *E. coli*, but such contributions are unlikely since we failed to detect any differences in the distribution and amounts of mucosal immune cells between WT and ST mice prior to DSS-induced colitis[18]. Therefore, we conclude that the proliferation of *E. coli* supported by α2,3-linked sialic acids was the main factor regulating the magnitude of intestinal inflammation triggered by DSS ingestion.

This study demonstrated the critical role of α2,3-linked sialic acids provided by milk-derived 3SL during lactation and by host mucosal glycans in establishing an intestinal niche for *E. coli*

in mice. Expansion of *E. coli* during colitis directly depended on sialic acid release from host glycans after sialidase activity. This resulting overgrowth of *E. coli* leads to exacerbation of the proinflammatory response by intestinal DCs. The beneficial outcome of sialidase inhibition on the severity of DSS-induced colitis suggests that sialidase inhibitors should be investigated as agents able to reduce intestinal inflammation by preventing dysbiosis manifested by *Enterobacteriaceae* expansion.

Materials and Methods

Bacterial DNA extraction and quantitative PCR

DNA was isolated from fecal samples using the QIAamp DNA Stool Mini Kit (Qiagen) according to manufacturer's instructions. Lysis temperature was increased to 95°C for 5 min to ensure complete cell lysis of Gram-positive cells. The proportion of bacterial family and genera in fecal samples were determined by real-time PCR using the EvaGreen qPCR Master Mix (Biotium). Cycling conditions were 40 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 25 s after an initial denaturation at 95°C for 3 min. Primer pairs specific for 16S rRNA of *Bacteroidetes* (Bac32F, Bac303R), *Enterobacteriaceae* (Eco1457F, Eco1652R), and total bacteria (Eub338F, Eub518R) were described previously[44-45]. Primers (UAL1939b, UAL2105b) targeting β-glucuronidase *uidA* gene was used to evaluate the relative abundance of *E. coli*[22]. Quantification values were calculated by the $2^{-\triangle Ct}$ method relative to total bacterial 16S rRNA amplicons[46].

16S rRNA pyrosequencing

Fecal DNA was isolated from fresh stool samples of 7-week old WT and ST male mice before and at day 8 after initiation of DSS treatment. The 16S rRNA V5-V6 region was amplified from fecal DNA samples using primer 784F and 1061R[47]. Amplicons were sequenced using a Roche 454 GS-FLX system (DNA Vision, Belgium). The QIIME software was used for taxonomic classification. Taxonomy was assigned using Ribosomal database Project (RDP) classifier and the Greengenes database[48]. Bacterial diversity was determined at the phylum, family and genus levels.

Mouse models

Wildtype and sialyltransferase *St3gal4*$^{-/-}$ mice[49] were of C57BL/6 background and derived from the same breed and maintained in light-cycled and climate-controlled facility. Animals were received regular laboratory chow diet (KLIBA extrudat No. 3436, Provimi Kliba, SA Switzerland) and sterile water *ad libitum*. Synchronized matings were set up for WT and ST mice to allow the exchange of newborn mice for cross-fostering experiments. All experiments were performed in compliance with the Swiss Animal Protection Ordinance and approved by the Veterinary Office "of the Canton of Zurich, Switzerland.

Antibiotic treatment and DSS induced colitis

Six to seven week-old, male mice were treated with 3-3.5% (w/v) DSS (MW = 36-50 kDa; MP Biomedicals) in drinking water for 5 days, followed by a supply of normal water until sacrifice of the animals. Body weight and physical activity were monitored daily. For long term antibiotic treatment, 3-week-old mice were provided with sterile drinking water supplemented with vancomycin (0.5 g L^{-1}), streptomycin (1 g L^{-1}), neomycin (1 g L^{-1}), chloramphenicol (0.5 g L^{-1}) or metronidazole (1 g L^{-1}) plus aspartame (0.25%, w/v) for 3 weeks prior to the beginning of DSS treatment.

Transepithelial permeability assay

Mice were gavaged with 600 mg kg^{-1} body weight of FITC-dextran (MW 3000-5000, Sigma) and whole blood was collected by cardiac puncture 4 h after gavage. Blood serum was collected after centrifugation at 1500 *x g* for 10 min. Serum fluorescence intensity was measured using a multi-detection microplate reader (Tecan Infinite M200 Pro, Switzerland) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. FITC concentration (µg ml^{-1}) was calculated from a standard curve using serial dilutions of FITC-dextran[50].

Bacterial strains and mutant construct

E. coli strain EHV2 was isolated from inflamed colon surface of DSS-treated C57/BL6 mice. The isolate strain was confirmed by 16S rRNA sequencing, universal stress protein *uspA*[23], gyrase *gyrB*[24] sequencing, and phenotypic analysis (API-20E Enterobacteriaceae identification kit; bioMerieux). *E. coli* strain HS996 was obtained from Genebridge and transformed with pET16b vector containing an ampicillin resistance gene. HS996-*nanT* knockout mutant was constructed by introducing a kanamycin-resistance cassette by homologous recombination into the nanT locus with followed by manufacture's instruction (Genebridge, Germany). The genomic primers used for the targeting construct were designed according to the flanking regions of the *E. coli nanT* gene: forward primer f182 was 5'-ATA CCA AAG CGT GTG GGC ATC GCC CAC CGC GGG AGA CTC ACA ATG AGT ACA ATT AAC CCT CAC TAA AGG GCG-3', and reverse primer r1723 was 5'-GCA ACA GGA TTA ACT TTT GGT TTT GAC TAA ATC GTT TTT GGC GCT GCC AAT AAT ACG ACT CAC TAT AGG GCT C-3'. Null mutation at the *nanT* locus was confirmed by genome sequencing and growth phenotype. *B. thetaiotaomicron* (DSM 2079^T) was obtained from the German collection of microorganisms and cell cultures (Braunschweig, Germany). Cells were grown in YCFA medium containing volatile fatty acids[51] at 37°C anaerobically in rubber-sealed Hungate tubes. Cell density was measured by a spectrophotometer at 600nm (S2100 Diode array, Biochrom WPA).

Carbohydrate metabolism assay

E. coli EHV2 (10^7 cells) were cultured in 3 ml of M9 minimal medium[52] containing 10 mM of either glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), fucose (Fuc) and *N*-acetylneuraminic acid (Sia) as single carbohydrate source at 37°C for 24 h. The milk oligosaccharides 3SL and 6SL were tested as 5 mM in 3 ml of M9 minimal medium supplemented with phosphate buffered saline (PBS) or sterile filtered mouse caecal fluid (1.5%, v/v). All neutral monosaccharides were purchased from Sigma-Aldrich except *N*-acetylneuraminic acid from Carbosynth (Berkshire, UK).The oligosaccharides 3SL and 6SL were obtained from Glycom A/S (Lyngby, Denmark). Cell density was determined by OD 600 values at 3, 6, 12 and 24 h. For determination the growth

phenotype in nanT mutant and ManX mutant, parental and mutant strains were cultured in modified M9 minimal medium (additional 0.003% L-Histidine, 0.004% Leucine, and 0.01% Yeast extract) containing 10 mM of glucose or *N*-acetylneuraminic acid.

Sialidase activity assay

Mouse caecal content was collected and centrifuged at 15'000 *x g* for 10 min at 4°C. The supernatant was filtered through a 0.45 µm membrane to yield caecal fluid. The fluorogenic substrate 2'-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid sodium salt (4-MU-NeuNAc; Carbosynth) was used to determine sialidase activity. In brief, caecal fluid (10%, v/v) was incubated with 0.1 mM 4-MU-NeuNAc in 0.2 ml of 100 mM sodium acetate buffer (pH 7.4) at 37 °C for 15 min. Assays were stopped by adding 0.8 ml of 0.5 M sodium carbonate buffer (pH 10.5) and further diluted 20-fold prior to fluorescence measurement. Cleaved 4-methylumbelliferone (4-MU) was measured by fluorescence detection in a multi-detection microplate reader at an excitation wavelength of 360 nm and an emission wavelength of 440 nm[53].

Quantitative PCR of bacterial sialidase genes

Sequences of sialidase (EC 3.2.1.18) genes from *Bacteroidaceae* were retrieved from the GH33 sialidase family of the Cazy database. Primers encompassing conserved DNA stretches of sialidase genes from *P. distasonis*, *B. vulgatus*, *B. thetaiotaomicron*, *B. fragilis* were designed based on multiple alignment analysis. The lack of significant sequence similarity of the selected primers with unrelated bacterial sequences was confirmed by BLAST analysis. The *B. vulgatus* sialidase primers used for quantitative PCR analysis were Bv-f266: GGAGGGGAAAGACTTATTTTGC, Bv-r501: TTCCACCACTTCTGCCGAC; Cycling conditions were 40 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 25 s after an initial denaturation at 95°C for 3 min. Quantification values are represented as gene copy numbers per µg of total fecal DNA.

Molecular cloning and purification of sialidase

The gene encoding *B. vulgatus*_4143 sialidase (GeneID: 5305102) was amplified by PCR using the genomic DNA from caecum sample in WT-DSS mouse as template. *Nde*I and *Bam*HI sites were introduced in the forward Bvu_4143F 5'- GGCCATATGAGAAACCCTAGCTTATTA -3' and reverse primer Bvu_4143R: 5'- GCGGGATCCTTATTTGGTCTTAATAAT -3', respectively. PCR conditions were thirty cycles of 30 s at 95°C, 30 s at 53°C, 3.5 min at 72°C. The PCR product was digested with *Nde*I and *Bam*HI and subcloned into the pET16b expression vector (Novagen). The pET16b- BVU_4143 vector was transformed into *E. coli* BL21-star (DE3) cells (Invitrogen) cultured in LB broth and supplemented with ampicillin (100 µg ml^{-1}) at 37°C. On reaching an OD_{600} value of 0.5, BVU_4143 expression was induced by adding 0.5 mM IPTG and incubating bacteria at 30°C for 5 h. Bacteria were then resuspended in 100 mM Tris-HCl plus 20 mM imidazole (pH 7.4) and disrupted by sonication. The resulting cell extract was incubated with Ni-sepharose (GE Healthcare Life Science) at 4°C overnight, washed with 100 mM Tris-HCl containing 40 mM imidazole and the His_6-tagged BVU_4143 sialidase was eluted with 500 mM imidazole.

Bacterial colonization fitness assay

The relative fitness of *E. coli* strains for colonization of the mouse intestine was monitored as described[34]. Briefly, 6 week-old WT mice were given drinking water containing ampicillin (2 mg ml^{-1}) for 2 days prior to gavage with 10^6 colony-forming units of parental HS996-amp^r and HS996-nanT mutant::$Kana^r$ *E.coli*. Fresh fecal samples were collected, serially diluted and plated on LB agar containing ampicillin (100 µg ml^{-1}) or ampicillin plus kanamycin (30 µg ml^{-1}) by day 1, 2, 5 and 10 after gavage. Competitive index was calculated as the ratio of parental to *nanT* mutant *E. coli*.

Quantification of caecal sialic acids

Mouse caecal content (approximately 500 mg) was weighed out and centrifuged for 10 min at 16,000 x g at 4°C. The supernant was collected and filtered through 0.45 µm cronus HPLC

membrane to get caecal fluid and stored at -20°C before use. Caecal fluid was derivatized with 1,2-diamino-4,5-methylene-dioxybezene (DMB; Sigma-Aldrich) as described previously[54]. In brief, 10 µl of caecal fluid was incubated with 200 µl of the DMB solution at 50°C for 2.5 h in the dark. DMB solution was prepared by dissolving DMB dihydrochloride (7 mM) in 1.4 M acetic acid containing 0.75M β-mercaptoethanol and 18 mM sodium hydrosulfite. The reaction was stopped by adding 800 µl of ice-cold distilled water. The derivatized product was analyzed by reverse-phase HPLC using a ODS Hypersil 150 x 3 mm column (Thermo scientific). The mobile phase was acetonitrile:methanol:water (9:7:84, v/v) at a flow rate of 0.3 ml min^{-1}. Florescence of the derivatized product was monitored at 373nm (excitation) and 448nm (emission). DMB-derivatized Neu5Ac was identified by comparison with authentic sialic acid standards.

Sialidase inhibition

The sialidase inhibitor *N*-acetyl-2,3-didehydro-2-deoxyneuraminic acid (Neu5Ac2en) was prepared in house based on published procedures[55]. For *in vitro* inhibition, *E. coli* EHV2 was cultured for 24 h at 37°C in M9 minimal media containing 5mM 3SL, caecal fluid (1.5%, v/v) and varying Neu5Ac2en concentrations. For *in vivo* inhibition, mice were anesthetized by isoflurane inhalation, gavaged with 300 µl of Neu5Ac2en (10 mg kg^{-1} per day) in sterile saline at days 0, 1, 2, 5 of DSS challenge. Control groups received sterile saline.

Histological staining of colonic tissue

Distal colons were removed, cut longitudinally, and fixed in 10% neutral buffered formalin then embedded in paraffin. Tissue samples were cut in serial 5-µm sections, which were stained with hematoxylin-eosin (Sigma-Aldrich). Histological sections were examined by using microscope Zeiss Axio Imager.Z2, objectives (Zeiss EC Plan – Neofluar 10x /0,3). Image was acquired by Zeiss AxioCam HrC camera and analyzed with Zeiss AxioVision software (AxioVs40V4.8.2.0). Sections were scored individually by an independent investigator blinded to the type of treatment. Morphological changes and leukocyte infiltration in the colon were scored as previously described[56]. The total score is represented as the sum of the epithelium

and infiltration score.

DC Isolation and stimulation

Mesenteric lymph nodes were isolated and incubated in 2.5 mg $ml^{-1}$of collagenase type D (Roche) in RPMI 1640 containing 10% FCS for 10 min at 37°C. Tissues were gently homogenized by passing through an 18-gauge needle, then incubated for 30 min at 37°C. The resulting cell suspensions were filtered through 40 µm cell strainers and incubated with Fc-blocker (anti-CD16/32) (eBioscience) for 10 min. $CD11c^+$ cells were isolated with anti-CD11c MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. $CD11c^+$ DCs (2×10^5 cells per ml) were culture in RPMI 1640 containing 10% FCS, and stimulated with fixed bacteria or PBS for 14 h at 37°C. *E. coli* (EHV2) and *B. thetaiotaomicron* (DSMZ 2079T) were fixed in 0.5% paraformaldehyde for 15 min at room temperature and washed with PBS prior to stimulation. Fixed bacteria were co-cultured with dendritic cells in a ratio 100:1. After stimulation, dendritic cells were stained with fluorochrome-labeled anti-mouse antibodies: MHCII-FITC (BD Bioscience), CD86-PE (eBioscience), CD40-PE-Cy7 (Biolegend), and CD11C-APC (BD Bioscience) for 30 min at 4°C. Cells were analyzed by flow cytometry (FACScanto II). For monocytes stimulation, human THP-1 cells ($1x10^6$ cells per ml) were stimulated with bacteria or PBS for 14 h as described above, but in bacteria to cells ratios of 5:1 and 1:1. After stimulation, THP-1 cells were stained with CD54-PE (BD Bioscience) for 30 min at 4°C and analyzed by flow cytometry. The supernatants of stimulated DCs were collected and analyzed for IL-6, IL-10, IL-12p40, and TNF-α cytokine production by multiplex bead array (Cytolab AG, Switzerland).

Statistical analysis

Results are presented as mean ± SEM unless specified. Difference between groups was analyzed by unpaired Student's t-test (two-tailed) and one-way ANOVA with Bonferroni's multiple comparison post-test using GraphPad Prism 5. P values below 0.05 were considered significant.

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Author contributions

T.H. and Y.L.H. designed the study; Y.L.H. performed the experiments; M.H. performed histological evaluation; M.v.I. provided sialidase inhibitors; T.H., Y.L.H., and C.C. analyzed data; all authors discussed results and wrote the manuscript.

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Figure legends

Figure 1. Bacterial composition of mice in DSS-induced colitis. (a) 16S rRNA pyrosequencing analysis of fecal microbial taxa families in control and DSS-treated WT and ST mice (at day 8 after DSS addition). **(b)** Pyrosequencing analysis of fecal microbial taxa in the genus level. Data show the average percentage of total identified sequences obtained from a pool of 8 mice per group. Only the bacterial taxa representing at least 1% of total identified sequences are presented.

Figure 2. Antibiotics effect on DSS-induced colitis. (a) Mice were treated with vancomycin (Van), neomycin (Neo), penicillin (Pen), streptomycin (Stp), chloramphenicol (CAM), and metronidazole (Met) for 3 weeks before DSS challenge for 5 days. Body weight was measured by day 8 after initiation of DSS challenge and given as percentage to the body weight of mice challenged with DSS without antibiotics. The data is represented as mean ± SEM. N=6-8, *$p < 0.05$ (two-tailed Student's t-test) **(b)** Intestinal permeability was measured by FITC-dextran levels in serum from control, colitogenic mice on day 5 of DSS challenge, and 3 weeks of antibiotic pre-treated mice. N=5, *$p < 0.05$ (ANOVA, Bonferroni's multiple comparison test) **(c)** Relative change in body weight of WT and ST mice treated with 0.5 g L^{-1} vancomycin (Van) for 7 days and 3% DSS for 5 days; control mice received DSS without vancomycin. **(d)** Relative change in body weight of WT and ST mice treated with 1 g L^{-1} streptomycin for 8 days and 3% DSS for 5 days. **(e)** Colon length was determined at the endpoint of DSS treatment. In (c-e) the data is represented as mean ± SEM from two independent experiments, N=6-8, *$p < 0.05$ (ANOVA, Bonferroni's multiple comparison test). **(f)** 16S rRNA pyrosequencing analysis of fecal microbial taxa families in untreated WT mice, in vancomycin (Van), streptomycin (Stp)-treated WT mice, and DSS challenged WT mice (WT-DSS). The data is represented as the percentage of total identified sequences obtained from a pool of 8 mice per group.

Figure 3. Intestinal *E. coli* in mice. Relative abundance of *E. coli* was determined by real-time PCR using specific u*idA* primers and quantified using the $2^{-\triangle Ct}$ method. **(a)** Relative abundance of *E. coli* in groups of 6 week-old WT and ST mice treated with the antibiotics vancomycin (Van) and streptomycin (Stp) and challenged with 3% DSS. Mice were received

with or without 8 days of antibiotic treatment and DSS for the first 5 days in drinking water. Control mice received sterile drinking water. **(b)** Relative abundance of *E. coli* in 6 week-old WT, ST and respectively cross-fostered (XF) mice, which were fed by foster mothers of the other genotype during lactation. The data is represented as median value. Each point indicates a single mouse from two independent experiments. N=6-8, *p < 0.05 (ANOVA, Bonferroni's multiple comparison test).

Figure 4. Sialic acid processing and uptake by *E. coli* in vitro. (a) Growth of *E. coli* EHV2 in M9 minimal medium containing single monosaccharide at 10 mM. Sia, *N*-acetylneuraminic acid, Glc, glucose, GlcNAc, *N*-acetylglucosamine, GalNAc, *N*-acetylgalactosamine, Gal, galactose, Fuc, fucose. **(b)** Growth of *E. coli* in minimal medium containing 5mM of 3SL and 6SL with and without supplementation of caecal fluid (CF, 1.5%, v/v) derived from WT mice. **(c)** Caecal fluid of conventional and DSS-challenged WT and ST mice were collected. Sialidase activity was determined by measuring fluorescent 4-MU-NeuNAc at 440 nm. **(d)** Sialidase activity was also measured in the caecal fluid of WT and WT mice treated with either streptomycin (Stp 1 g L^{-1}), vancomycin (Van 0.5 g L^{-1}), or antibiotic cocktail (AVMN: ampicillin, vancomycin, metronidazole, and neomycin). In (c-d) the data is represented as mean ± SEM from two independent experiments, N=5-8, *p < 0.05 (two-tailed Student's t-test). **(e)** The abundance of the *B. vulgatus* BVU_4143 sialidase gene was determined by real-time PCR from caecum samples of WT and ST mice, and **(f)** caecum samples of antibiotic treated WT mice. The data is represented as gene copy number per µg of fecal DNA. In (e-f) each data point indicates a single mouse, N=5-7, * p < 0.05 (two-tailed Student's t-test).

Figure 5. Sialic acid catabolism is required for maintaining *E. coli* colonization. (a) Growth of parental *E. coli*, *nanT* mutant, and *ManX* mutant in modified minimal media containing 10 mM of glucose or sialic acid. **(b)** Relative fitness of parental *E. coli* and *nanT* mutant in colonization of WT mice and **(c)** ST mice. Bacterial colonization fitness were determined by counting colony forming unit (cfu) of serial diluted fecal feces collected at indicated time points. In (b-c) data are shown as median ± interquartile range. N =8, *p < 0.05 (two-tailed Student's t-test). **(d)** Competitive index was calculated as ratio of parental *E. coli* to *nanT* mutant in WT and ST mice over 10 days after administration. The data is represented as

median value, and each dot indicates an individual animal. N =8, *p < 0.05 (two-tailed Student's t-test). **(e)** Levels of free Neu5Ac in the caecal fluid of control and DSS-challenged WT mice were determined by HPLC analysis. **(f)** Levels of free Neu5Ac in the caecal fluid of control (C), streptomycin-, and vancomycin-treated mice measured 5 days post treatment. In (e-f) the data is represented as mean ± SEM from two independent experiments. N=6-8, *p < 0.05 (ANOVA, Bonferroni's multiple comparison test).

Figure 6. Reduced intestinal *E. coli* expansion by sialidase inhibition. (a) *In vitro* growth of *E. coli* EHV2 in minimal media containing 10 mM Glc or 3SL and the presence of caecal fluid and inhibitor Neu5Ac2en (0, 20, 50, 100 µM). **(b)** Levels of sialic acid in the caecal fluid of control, and inhibitor-treated WT mice were measured by HPLC analysis. control mice were administered with sterile PBS. In (a-b) the data is represented as mean ± SEM from two independent experiments, N=5, *p < 0.05 (two-tailed Student's t-test). **(c)** Relative abundance of intestinal *E. coli* in control (PBS-treated) and Neu5Ac2en-treated WT mice (10 mg kg^{-1} per day) was determined at day 8 after initiation of DSS challenge. **(d)** Relative change in body weight of control and Neu5Ac2en-treated WT mice during DSS challenge for 5 days. Arrowheads indicate the time points of Neu5Ac2en administration. **(e)** Colon length was measured at day 8 after initiation of DSS challenge. In (d-e) the data is represented as mean ± SEM from two independent experiments, N=6-8, *p < 0.05 (two-tailed Student's t-test). **(f)** Representative histological sections of colon tissues from untreated WT mice (Mock), DSS-treated mice (DSS), and DSS-treated mice administered with sialidase inhibitor (DSS/Neu5Ac2en). Arrowheads indicate infiltrating leukocytes. Scale bar, 100 µm. **(g)** Scoring of colitis severity by quantitative examination of tissue alteration and leukocytes infiltration.

Figure 7. Stimulation of dendritic cells and monocytes. (a) Bacteria were fixed with 0.5% paraformaldehyde in PBS, washed and co-cultured at a ratio of 100:1 with mouse mesenteric $CD11c^{+}$ DCs. Ec, *E. coli* EHV2; Bt, *B. thetaiotaomicron*. Cell surface expression of CD86, CD40 and MHC-II was analyzed by flow cytometry. **(b)** Human monocytic THP-1 cells were stimulated with fixed bacteria at ratios of 5:1 and 1:1 to cells for 14 h at 37°C. Cell surface expression of CD54 was analyzed by flow cytometry. In (a-b) the data is represented as mean fluorescence intensity MFI ± SEM from three independent experiments. N=6, *p <0.05.

(ANOVA, Bonferroni's multiple comparison test). **(c)** Cytokine expression in the culture supernatant of stimulated mesenteric $CD11c^+$ DCs stimulated for 14 h at 37°C with fixed *E. coli* (Ec), *B. thetaiotaomicron* (Bt), and 500 ng ml^{-1} of LPS. PBS was used as negative stimulation control. Data is represented as mean ± SEM. N= 4-5, *p < 0.05 (ANOVA, Bonferroni's multiple comparison test).

Figure 1

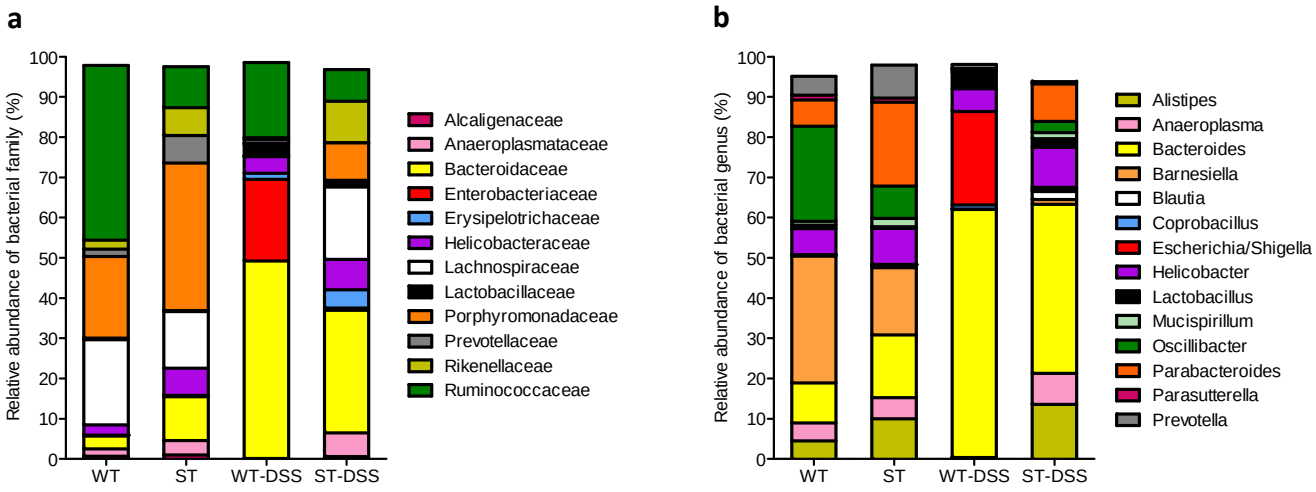

a
Relative abundance of bacterial family (%)
100
90
80
70
60
50
40
30
20
10
0
WT
ST
WT-DSS
ST-DSS
Alcaligenaceae
Anaeroplasmataceae
Bacteroidaceae
Enterobacteriaceae
Erysipelotrichaceae
Helicobacteraceae
Lachnospiraceae
Lactobacillaceae
Porphyromonadaceae
Prevotellaceae
Rikenellaceae
Ruminococcaceae
b
Relative abundance of bacterial genus (%)
100
90
80
70
60
50
40
30
20
10
0
WT
ST
WT-DSS
ST-DSS
Alistipes
Anaeroplasma
Bacteroides
Barnesiella
Blautia
Coprobacillus
Escherichia/Shigella
Helicobacter
Lactobacillus
Mucispirillum
Oscillibacter
Parabacteroides
Parasutterella
Prevotella

Figure 2

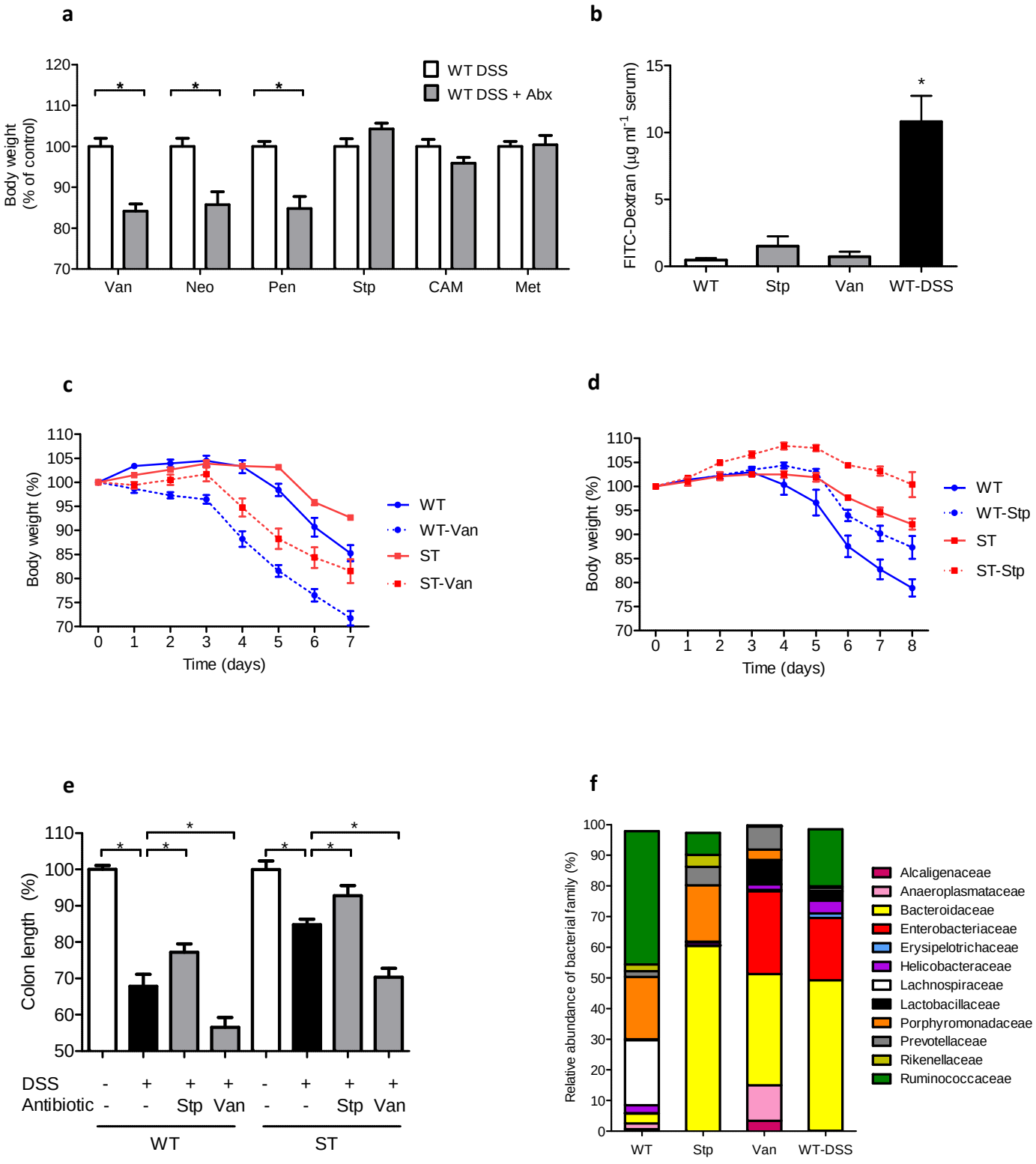

a
Body weight (% of control)
WT DSS
WT DSS + Abx
Van
Neo
Pen
Stp
CAM
Met
b
FITC-Dextran (µg ml-1 serum)
WT
Stp
Van
WT-DSS
c
Body weight (%)
Time (days)
WT
WT-Van
ST
ST-Van
d
Body weight (%)
Time (days)
WT
WT-Stp
ST
ST-Stp
e
Colon length (%)
DSS
Antibiotic
Stp
Van
WT
ST
f
Relative abundance of bacterial family (%)
Alcaligenaceae
Anaeroplasmataceae
Bacteroidaceae
Enterobacteriaceae
Erysipelotrichaceae
Helicobacteraceae
Lachnospiraceae
Lactobacillaceae
Porphyromonadaceae
Prevotellaceae
Rikenellaceae
Ruminococcaceae
WT
Stp
Van
WT-DSS

Figure 3

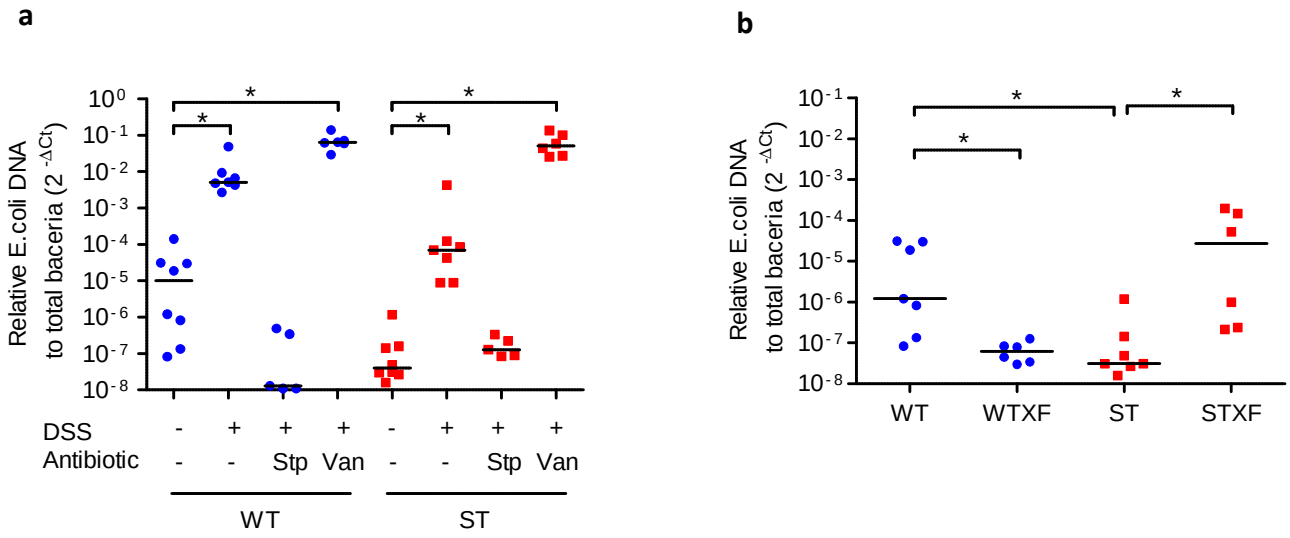

a
b
Relative E.coli DNA
to total baceria (2 -ΔCt)
DSS
Antibiotic
Stp
Van
WT
ST
WTXF
STXF

Figure 4

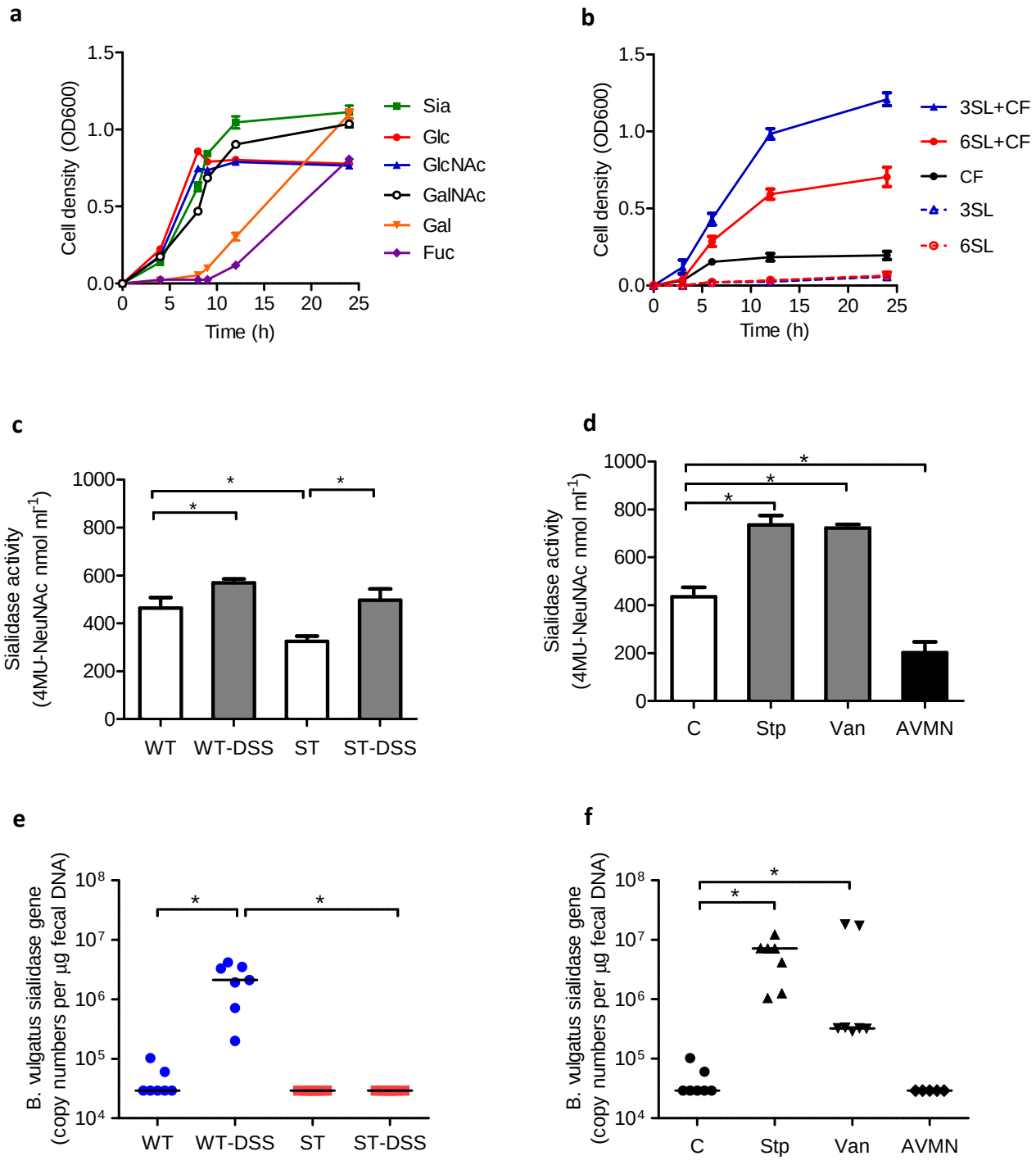

a
Cell density (OD600)
Time (h)
Sia
Glc
GlcNAc
GalNAc
Gal
Fuc
b
Cell density (OD600)
Time (h)
3SL+CF
6SL+CF
CF
3SL
6SL
c
Sialidase activity (4MU-NeuNAc nmol ml-1)
WT
WT-DSS
ST
ST-DSS
d
Sialidase activity (4MU-NeuNAc nmol ml-1)
C
Stp
Van
AVMN
e
B. vulgatus sialidase gene (copy numbers per µg fecal DNA)
WT
WT-DSS
ST
ST-DSS
f
B. vulgatus sialidase gene (copy numbers per µg fecal DNA)
C
Stp
Van
AVMN

Figure 5

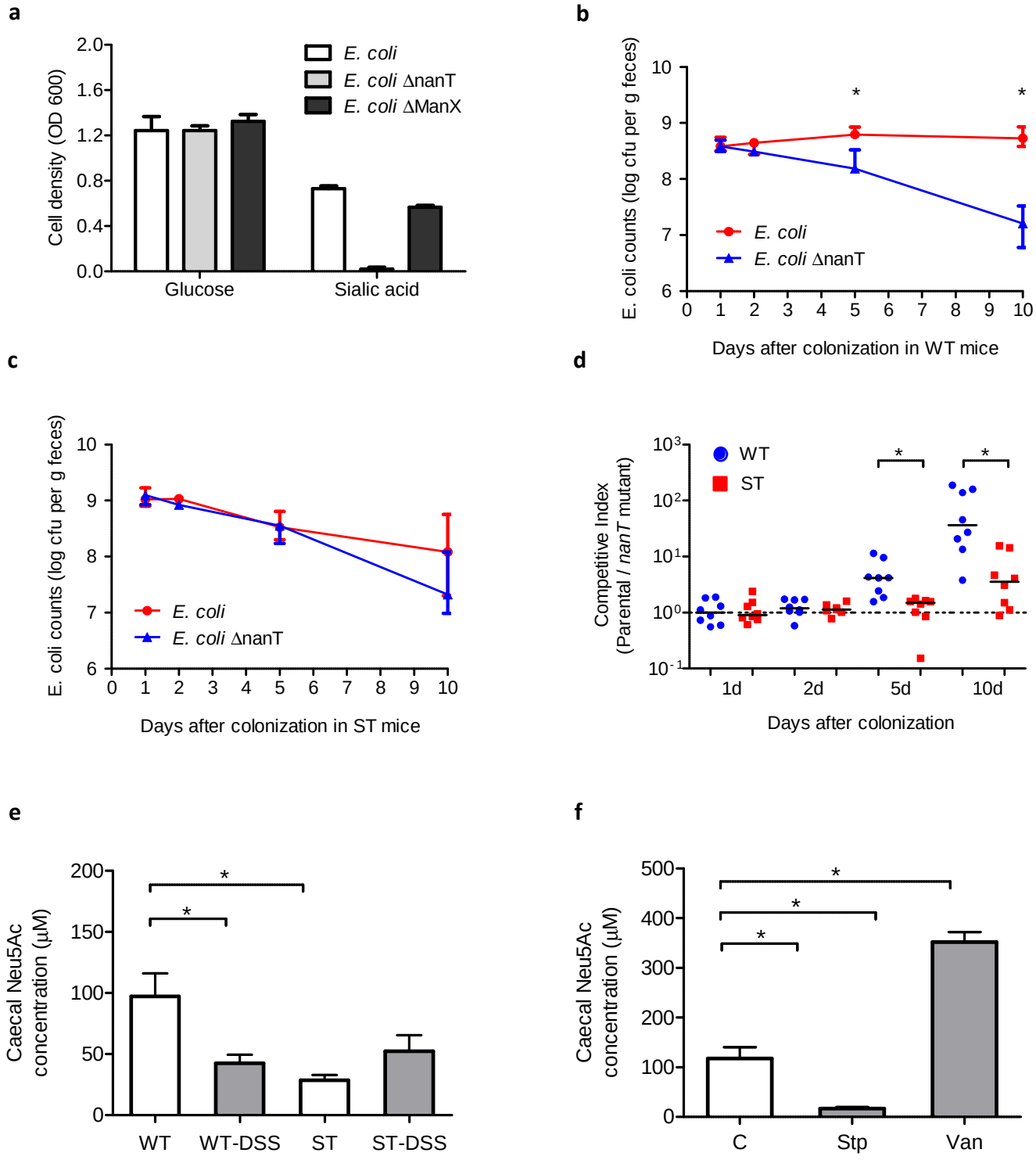

a
Cell density (OD 600)
2.0
1.6
1.2
0.8
0.4
0.0
E. coli
E. coli ΔnanT
E. coli ΔManX
Glucose
Sialic acid
b
E. coli counts (log cfu per g feces)
Days after colonization in WT mice
E. coli
E. coli ΔnanT
c
E. coli counts (log cfu per g feces)
Days after colonization in ST mice
E. coli
E. coli ΔnanT
d
Competitive Index
(Parental / nanT mutant)
WT
ST
1d
2d
5d
10d
Days after colonization
e
Caecal Neu5Ac concentration (μM)
WT
WT-DSS
ST
ST-DSS
f
Caecal Neu5Ac concentration (μM)
C
Stp
Van

Figure 6

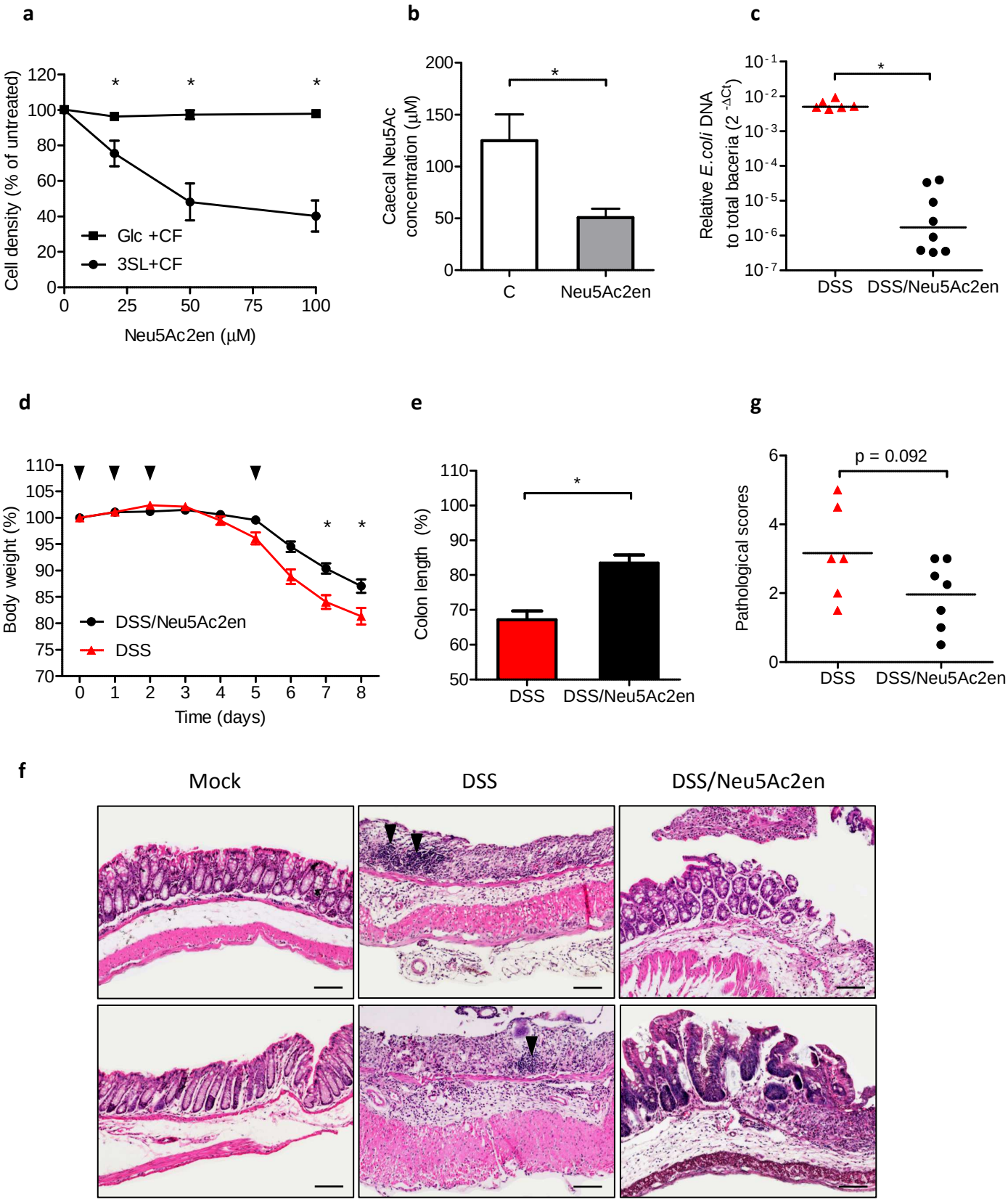
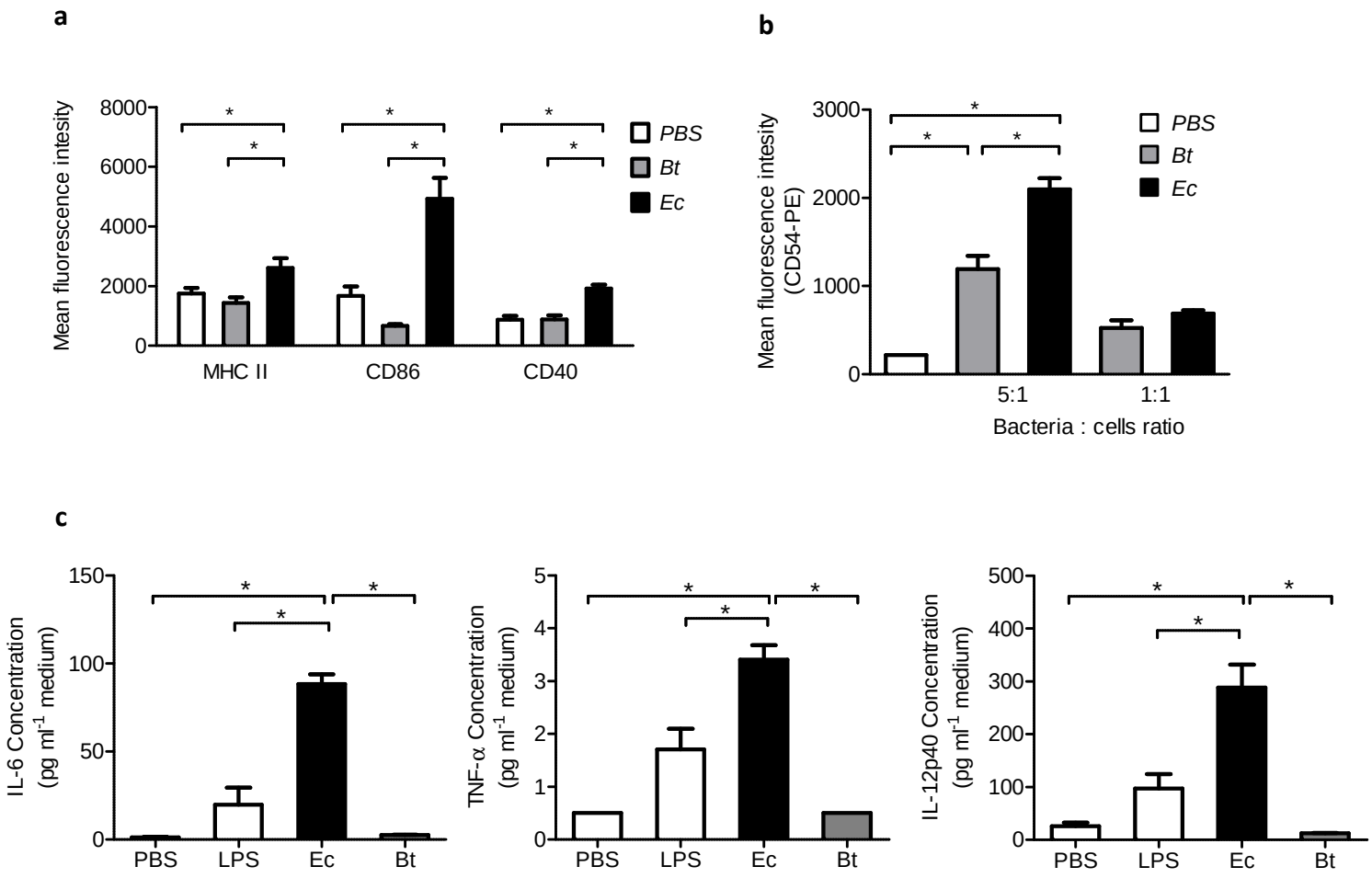
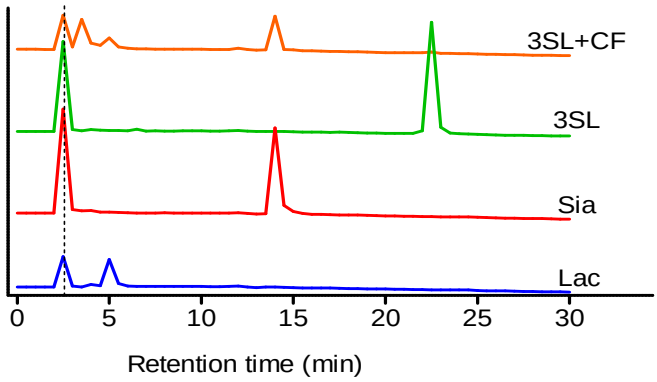

a
Cell density (% of untreated)
Glc +CF
3SL+CF
Neu5Ac2en (µM)
b
Caecal Neu5Ac concentration (µM)
C
Neu5Ac2en
c
Relative E.coli DNA to total baceria (2 -ΔCt)
DSS
DSS/Neu5Ac2en
d
Body weight (%)
DSS/Neu5Ac2en
DSS
Time (days)
e
Colon length (%)
DSS
DSS/Neu5Ac2en
g
p = 0.092
Pathological scores
DSS
DSS/Neu5Ac2en
f
Mock
DSS
DSS/Neu5Ac2en

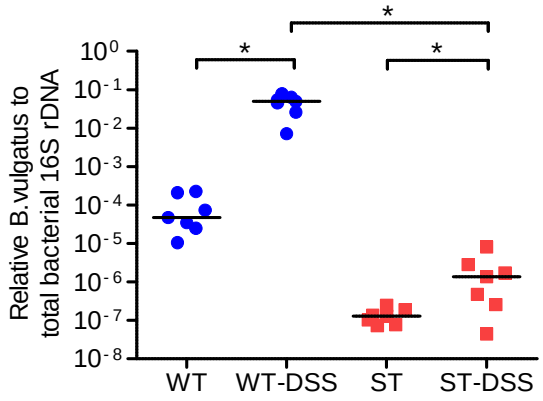
Figure 7


a
Mean fluorescence intesity
8000
6000
4000
2000
0
MHC II
CD86
CD40
PBS
Bt
Ec
b
Mean fluorescence intesity (CD54-PE)
3000
2000
1000
0
5:1
1:1
Bacteria : cells ratio
PBS
Bt
Ec
c
IL-6 Concentration (pg ml-1 medium)
150
100
50
0
TNF-α Concentration (pg ml-1 medium)
5
4
3
2
1
0
IL-12p40 Concentration (pg ml-1 medium)
500
400
300
200
100
0
PBS
LPS
Ec
Bt

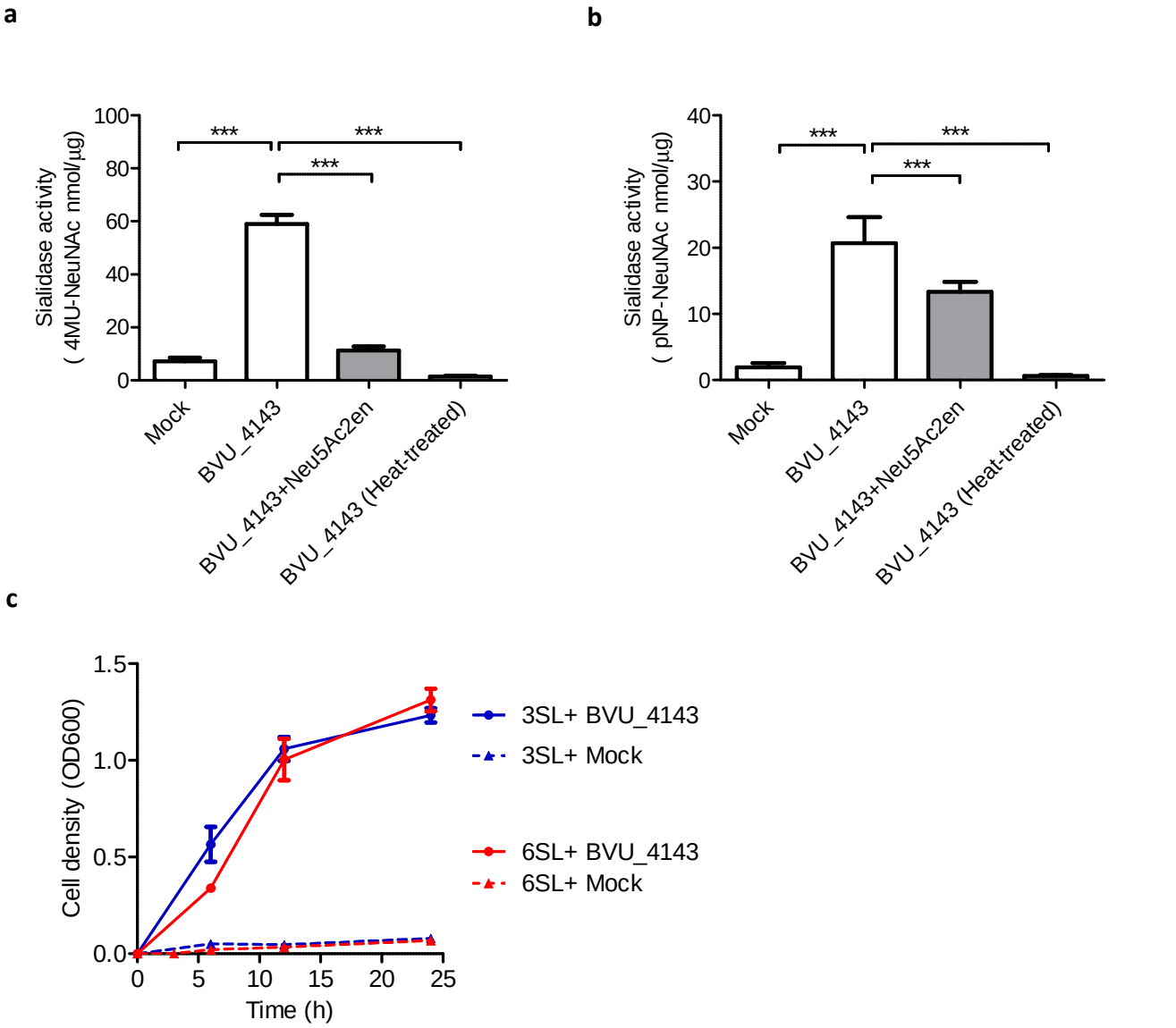
Supplementary Information



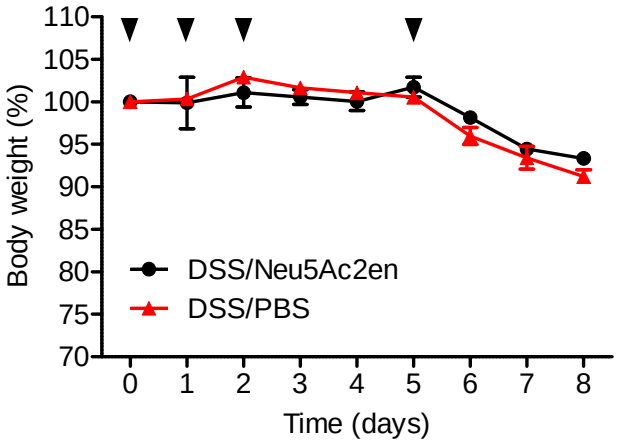
Supplementary Figure 1. Specificity of mouse caecum fluid sialidase. Cleavage of sialic acid from 3SL after incubation with caecum fluid for 16 h at 37°C. Reaction products were separated by HPLC using a CarboPac PA200 column (Dionex). Carbohydrates were identified by comparison with authentic standards. Lac, lactose; Sia, *N*-acetylneuraminic acid. Representative chromatograms from two independent experiments are shown.



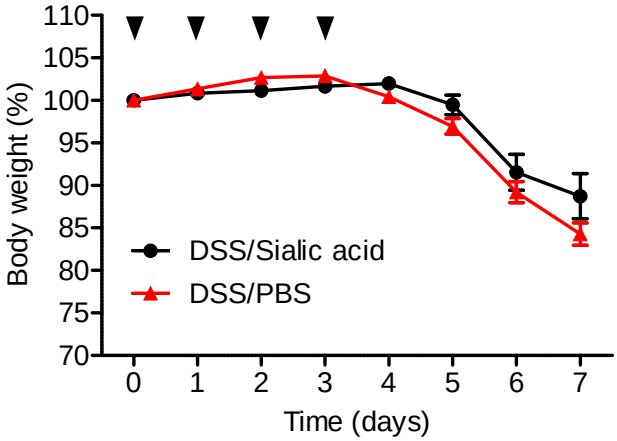
Supplementary Figure 2. Relative abundance of *B. vulgatus* in the colon of mice. The relative abundance of *B. vulgatus* was determined by real-time PCR using TaqMan assay. Each data point represents a single mouse from two independent experiments, N=6-7, *p < 0.05 (Two-tailed Student's t-test).



Supplementary Figure 3. Characterization of *B. vulgatus* BVU_4143 sialidase. (a) Recombinant BVU_4143 was purified as His-tagged protein. Sialidase activity of recombinant protein was determined by the hydrolysis of 4-MU-NeuNAc and **(b)** pNP-NeuNAc. For sialidase inhibition, B. vul sialidase was pretreated with Neu5Ac2en for 20 min with final concentration 200 µM. Heat, heat inactivation at 95 ^{o}C for 10 min. **(c)** Growth of *E. coli* EHV2 in minimal medium containing 5 mM of 3SL and 6SL with and without supplementation of recombinant sialidase. Data represent as mean ± SEM, N=4, ***p < 0.001 (Two-tailed Student's t-test).



Supplementary Figure 4. Administration of sialidase inhibitor in ST mice. ST mice were administrated with PBS or Neu5Ac2en (10 mg/kg/day) at indicated time points (arrowhead) during DSS (3%) challenge for 5 days. Relative change in body weight in ST mice challenged with DSS was monitored daily, N=4.



Supplementary Figure 5. Oral administration of sialic acid in WT mice. WT mice were orally gavaged with 300 µl of 100 mM sialic acid or sterile PBS at indicated time points (arrowhead) during DSS (3%, w/v) challenge for 5 days. Relative change in body weight was monitored daily, N=8, not significant (Two-tailed Student's t-test).

Supplementary Methods

Sialidase specificity assay

The linkage specificity of caecum sialidase was determined by incubating 0.1 ml of 100 mM 3SL, 100 mM 6SL with (15%, v/v) sterile caecum fluid in at 37°C for 16 h. Reaction products were separated by HPLC using a CarboPac PA200 column (Dionex) and detected by pulsed amperometry. The running conditions were 5 min of 100 mM NaOH for 5 min, followed by a linear gradient of sodium acetate from 0 to 250 mM over 40 min at a flow rate of 0.35 ml/min. The column was rinsed with 1 M sodium acetate for 5 min and equilibrated with 100 mM NaOH for 10 min. The retention times for lactose, sialic acid, 6SL, and 3SL were 4.7, 14.0, 22.0 and 22.4 min respectively. Sialic acid concentration was determined by comparison of peak area with an authentic standard solution.

Sialidase activity assay

The recombinant *B. vulgatus*_4143 sialidase was expressed and purified as previously described. The fluorogenic substrate (4-MU-NeuNAc; Carbosynth) was used to determine sialidase activity. In brief, recombinant sialidase (ca. 0.25 µg) was incubated with 0.1 mM 4-MU-NeuNAc in 0.2 ml of 100 mM sodium acetate buffer (pH 7.4) at 37°C for 15 min. Assay was stopped by adding 0.8 ml of 0.5 M sodium carbonate buffer (pH 10.5) and diluted 20-fold prior to fluorescence measurement at an excitation wavelength of 360 nm and an emission wavelength of 440 nm. 2-O-(4-Nitrophenyl)-α-D-N-acetylneuraminic acid (pNP-NeuNAc; Carbosynth) was also used to determine the sialidase activity. Recombinant sialidase was incubated with 200 µM pNP-NeuNAc in 0.2 ml of 100 mM Tris-Cl buffer (pH 7.4) at 37°C for 2 h. Assay was stopped by adding 0.2 ml of 0.4 M NaOH-glycine buffer (pH 10.8) and measured the absorption at 405nm with a microplate reader.

RESULTS : Manuscript 2

Butyrate-producing bacteria improve the course of colitis in mice challenged with dextran sulfate sodium

(Manuscript in preparation)

Yen-Lin Huang[1], Alfonso Die[2], Christophe Chassard[2],Thierry Hennet[1*]

[1]Institute of Physiology and Zurich Center of Integrative Human Physiology, University of Zurich, Zurich, Switzerland.

[2] Laboratory of Food Biotechnology, Institute of Food, Nutrition and Health, ETH Zurich, Zurich, Switzerland.

* Corresponding author: Thierry Hennet, Institute of Physiology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland.

Email : thennet@access.uzh.ch

Tel : +41 44 635 5080

Fax : +41 44 635 6814

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Abstract

The gut microbiota plays critical roles in the establishment and maintenance of intestinal health. Imbalance of the gut microbiota is a hallmark of intestinal inflammation. In this study, we investigate the changes affecting the microbiota in wildtype and antibiotic-treated mice, and attempt to correlate altered microbial metabolism with the severity of colitis. We identify a reduction and imbalance of SCFAs in mice challenged by DSS or subjected to selective antibiotic treatments. In particular, the caecal levels of butyrate in mice inversely correlate with disease development. We also show that intestinal inflammation correlates with loss of butyrate-producing bacteria, such as *Faecalibaterium prausnitzii* and *Eubacterium hallii.* Moreover, the beneficial effect of butyrate enema on the severity of colitis suggests that butyrate administration represents a valid therapeutic approaches for the treatment of inflammatory bowel disease.

Introduction

Inflammatory bowel disease (IBD), characterized by chronic inflammation of the gastrointestinal tract, is an emerging disease prevalent in developed countries. Of which, Crohn's disease (CD) and ulcerative colitis (UC) are the most common types of IBD. It is widely accepted that IBD is caused by an inappropriate immune response against the gut microbiota in a genetically susceptible individuals [1]. Studies of human IBD patients and animal IBD models suggested that dramatic alterations of microbial composition in the intestine contribute to disease development. Although accumulating studies pointed to the potential role of gut microbiota in mediating IBD, no specific pathogenic bacterium could be directly assigned as cause of this disease. Nevertheless, increased *Enterobacteriaceae* and decreased in members of *Clostridium* cluster IV and XIVa are reproducibly documented in IBD patients [2]. For instance, a reduction of *Faecalibacterium prausnitzii*, a member of *Clostridium* cluster IV is repeatedly observed in patients with CD and is associated with a higher risk of recurrence disease [3-4]. The anti-inflammatory effects of *F. prausnitzii* have been associated with their production of short-chain fatty acids such as butyrate [3, 5].

Butyrate, which is produced through bacterial fermentation of dietary carbohydrates in the intestine, exhibits a variety of functions in maintaining mucosal integrity and regulating immune responses [6]. Firstly, butyrate is a preferential energy source for colonocytes, mediating their proliferation and differentiation. Moreover, butyrate can strengthen the mucosal integrity by stimulating the production of mucins and antimicrobial peptides, and increasing the expression of tight junction proteins [6-7]. Notably, butyrate exerts a strong anti-inflammatory effect primarily through inhibition of histone deacetylase activity, resulting in a suppression of nuclear factor kappa B (NF-ƙB) activation and downstream pro-inflammatory signaling [8]. The multiple beneficial effects of butyrate on gastrointestinal physiology have made it an ideal candidate to treat IBD. Several clinical studies have revealed the beneficial effects of butyrate on attenuating intestinal inflammation in IBD patients [9-11]. However, a variety of other studies failed to show any clinical improvement [12-13] possibly because of differences in dosage, frequency, route and duration of administration.

Because of the low compliance in butyrate enema, administration of butyrate-producing bacteria enables the production of butyrate *in situ*, may become a potential treatment against IBD. Although microbial analysis has shown that the majority of butyrate-producing bacteria in human belong to *Clostridial* cluster IV and XIVa [14], the contribution of other butyrate-producing taxa is not fully elucidated. Therefore, we here investigate the interactions between gut microbiota, microbial metabolites, and the severity of colitis with the focus on identifying the roles of butyrate-producing species in a colitis model in mice.

Results

Antibiotics influence the severity of DSS-induced colitis in mice

To investigate the contribution of intestinal microbiota to colitis development, we have altered the intestinal microbial composition by selective antibiotic treatment in both wildtype (WT) and sialyltransferases *St3gal4*$^{-/-}$ (ST) mice. Mice were pre-treated with antibiotics for 3 weeks before being challenged with DSS to induce colitis. Vancomycin, neomycin and penicillin treatment exacerbated the severity of DSS-induced colitis in both WT and ST mice as shown by body weight loss compared to control group **(Fig. 1a-c)**. By contrast, streptomycin treatment attenuated body weight loss **(Fig. 1d)**. The protective effect of streptomycin towards DSS challenge was even more pronounced when streptomycin and DSS were administered in the same period in both WT and ST mice. Intestinal inflammation was further confirmed by measuring the colon length of antibiotic-treated mice. The shortening of colon length induced by DSS ingestion was aggravated by vancomycin, neomycin and penicillin, but unaffected by streptomycin **(Fig. 1e)**. To exclude any damaging effect caused by the use of antibiotics on intestinal barrier, we have previously examined the intestinal epithelial permeability by measuring the leakage of FITC-dextran in the bloodstream [15]. Among all the antibiotic-treated group, we did not detect any significant damage on the intestinal barrier function. Taken together, these results confirmed the contribution of specific groups of bacteria in influencing the severity of intestinal inflammation.

Effects of antibiotics on intestinal microbiota

To determine whether exposure to antibiotic influenced specific groups of gut microbiota, we examined the microbial diversity from fecal samples of control and antibiotic-treated mice. The microbial compositional change was compared by denature gradient gel-electrophoresis (DGGE) of group specific bacterial 16S rDNA. The DGGE profiles revealed that bacterial composition of WT mice varied significantly among different antibiotic treatments. Of which, vancomycin and penicillin treatment promoted the outgrowth of *Enterobacteriaceae* spp. and *Proteobacteria* spp. but reduced the abundance unclassified *Clostridial* cluster IV and XIVa. These findings were confirmed by real-time PCR analysis targeting *Clostridia* cluster IV **(Fig. 2a)**, *Lachnospiraceae* (cluster XIVa) **(Fig. 2b)**, *Ruminococcaceae* **(Fig. 2c)**, and *Enterobacteriaceae* **(Fig. 2d)**. By comparison, streptomycin exhibited a minor effect to the major bacterial taxa family but dramatically reduced *Enterobacteriaceae* levels **(Fig. 2d)**. Further analysis on bacterial genome suggested that *E. coli* accounted for the increased abundance of *Enterobacteriaceae* after vancomycin and penicillin treatment, which was consistent with our previous studies showing a positive correlation between the abundance of *E. coli* and the magnitude of inflammation [15].

To better characterize bacterial change at the species level, we combined *Bacteroidaceae*- and *Clostridial* cluster-specific PCR and DGGE analysis. In this approach, we identified an ubiquitous presence of *Bacteroides acidifaciens* among control and all the antibiotic-treated groups, whereas *Bacteroides vulgatus* was only detected after antibiotic or DSS treatment but not in control mice **(Supplementary Fig. 1)**. Moreover, *Bacteroides uniformis* and *Parabacteroides distasonis* were only identified in ST mice but not in WT mice **(Supplementary Fig. 1)**. The anti-inflammatory effect of *P. distasonis* has been demonstrated in a recent study showing that some bacterial membrane component attenuated murine colitis via inhibiting TNF-α production, which may explain the better protection of ST mice against DSS-induced colitis [16]. However, we do not find strong correlations between the predominant bacterial families, such as *Bacteroidaceae* and *Lactobacillaceae,* and the magnitude of inflammation **(Fig. 2e-f)**. Comparison studies on healthy subjects and IBD patients also yielded inconsistent results when associating major bacterial groups with

disease progression [17-18]. Therefore, to evaluate the functional impact of microbial change, we have also analyzed bacterial metabolites, thereby focusing on short-chain fatty acids (SCFAs), which are major fermentation products.

Caecal SCFAs influence colitis in mice

The levels of faecal SCFAs have been showed to be related with disease activity in human IBD. For instance, a significant reduction of butyrate was implicated with UC, whereas an increase of lactate correlated with diarrhea in UC patients [19]. Acetate and propionate were also found to be reduced in IBD compared to health individuals [20]. The anti-inflammatory properties of SCFAs have been demonstrated for butyrate and propionate, which induce the differentiation of T_{Reg} cells [21], and interact with G protein-coupled receptors to stimulate production of anti-inflammatory cytokines, such as IL-10 and TGF-β [22]. In this study, we found elevated levels of total caecal SCFAs in control WT mice compared to ST mice **(Fig. 3a, Table 2)**. However, when mice were challenged by DSS, the reduction of caecal SCFAs were more evident in WT mice, suggesting an imbalance of gut bacteria during intestinal inflammation. By contrast, ST mice maintained relatively stable levels of SCFAs before and after DSS-induced colitis, indicating a minor microbial change. Moreover, the levels of caecal SCFAs decreased markedly among all the tested antibiotic treatment groups compared to the control group **(Fig. 3b, Table 2)**. In particular, propionate and butyrate were completely absent in mice treated with vancomycin and penicillin, whereas lactate was increased compared to control mice, thus pointing to an inverse correlation between disease activity and the levels of SCFAs.

In addition to the absolute amounts of caecal SCFAs, molar ratio between major SCFAs reflected the balance or dysbiosis of microbiota in the intestine. The molar ratio of caecal acetate: propionate: butyrate in WT mice (3.3: 1: 1) was similar to that of ST mice (3.3: 1.3: 0.5) **(Fig. 3c).** However, when mice were challenged by DSS, the ratio of caecal SCFAs in WT-DSS mice skewed to 90: 9: 1 because of the significant decrease of butyrate production, whereas the ratio remained relatively stable in ST-DSS mice (5.9: 1.3: 1) **(Fig. 3c)**. Imbalance of caecal SCFAs was also confirmed in antibiotic-treated model. WT mice treated with

streptomycin showed stable levels of caecal SCFAs (3.1: 0.7: 1) and no significant impact on colitis development compared to control mice, whereas vancomycin- and penicillin-treated mice showed completed loss of caecal butyrate and propionate **(Fig. 3d)**. Overall, the levels of caecal butyrate inversely correlated with colitis development, which underlined the significance of butyrate-producing bacteria in maintaining colonic health.

Decreases of butyrate-producing bacteria in colitogenic mice

Because the levels of caecal butyrate varied significantly in healthy and inflamed mice, we evaluated whether a reduction of butyrate relates to decreased levels of butyrate-producing bacteria in mice. Based on phylogenetic studies of human microbiome, we targeted the bacterial groups of Clostridial clusters IV and XIVa, which include most butyrate-producing bacteria [23]. By analyzing Clostridial-specific DGGE profiles, we found a complete loss of cluster IV *Ruminococcus* and *Oscillibacter* spp. in WT mice after vancomycin and penicillin treatment **(Supplementary Fig. 2)**. In addition, faecal samples of ST mice revealed lower diversity of *Ruminococcus* such as *R. bromii* and *R. champanellensis* **(Supplementary Fig. 2)**. However, *Ruminococcus* spp. have not been shown to produce butyrate. Therefore we targeted known butyrate-producing bacteria, including the species related to *Eubacterium*, *Roseburia*, *Faecalibacterium* and *Coprococcus* [23]. A profound decrease of Clostridial cluster XIVa spp. **(Fig. 4a)** and *F. prausnitzii* **(Fig. 4b)** in antibiotic- and DSS-treated WT mice, confirmed the positive correlation of these butyrate producers and caecal butyrate levels in mice. We also quantified the abundance of major butyrate producers, *Roseburia* spp. and *Eubacterium hallii*. We observed that *Roseburia* spp. was only detected in WT control mice but not any other antibiotic-treated WT mice and ST mice **(Fig. 4c)**. The exclusive presence of *Roseburia* spp. in WT mice may explain the high caecal butyrate levels detected in WT mice, since these bacteria are considered to be as major butyrate producer [23]. By contrast, *E. hallii* was only identified in ST and DSS-treated ST mice but not in WT mice **(Fig. 4d)**, suggesting that this bacterium may contribute to the residual butyrate production (mean butyrate levels in ST mice: 9.3 mM; ST-DSS: 8.4 mM; WT-DSS: 0.4 mM) in ST mice, and that their abundance correlated with reduced inflammation in ST mice. Overall, our results

showed that intestinal inflammation matched with lower levels of mucosal butyrate-producing bacteria, particularly *F. prausnitzii* and *E. hallii.* The results were also consistent with recent studies indicating that altered mucosal layer and dysbiosis may impair the colonization fitness of these butyrate producers and further influence butyrate metabolism in the gut [3, 24].

Butyrate administration attenuates intestinal inflammation

The impact of butyrate on colonic health underlines its beneficial role against IBD. However, the delivery model of butyrate to particular colon regions remains problematic. Therefore, we compared the therapeutic effect of butyrate via different delivery modes in murine DSS-induced colitis model. Firstly, WT mice were orally supplemented with 100 mM sodium butyrate in drinking water for 7 days, during which they also challenged by DSS on the first 5 days. The water consumption and disease activity was monitored daily. WT mice treated in such a way with sodium butyrate showed no significant change of body weight compared to control mice treated with sterile water **(Fig. 5a)**. A similar outcome was also observed in ST mice **(Fig. 5b)**. In contrast to dietary butyrate-containing fibers which are slowly released along the gastrointestinal tract, solute sodium butyrate is directly available and immediately absorbed in the upper digestive tract before reaching the colon [7], which suggests that oral supplementation of butyrate salt may be impractical for clinical treatment. Next, we evaluated the effects of butyrate enema on attenuating intestinal inflammation by rectal administration of butyrate-containing saline during DSS-induced colitis. Mice treated with butyrate enema showed a reduced loss of body weight in the initial phase of colitis, but without reaching any significant improvement in the late-stage of colitis **(Fig. 5c)**. This limited efficacy may be explained by the short-term effect of butyrate enema (only once per day until day 4) as well as a discontinuous butyrate exposure of the colon. Nevertheless, the amelioration of the inflammation and symptoms such as reduced the shortening of colon **(Fig. 5d)**, and decreased bleeding (data not shown) strongly suggested the protective role of butyrate against colitis. Taken together, our results showed that butyrate enema has therapeutic effects, at least in part, against DSS-induced colitis. Further investigation should

be focus on improving the regimen, such as dosage, frequency, and duration as well as evaluating the capability of butyrate-producing bacteria, such as *F. prausnitzii* and *E. hallii* identified in this study, on ameliorating colitis.

Discussion

In the present study, we showed a significant reduction among members of the Clostridial cluster IV and XIVa in faecal samples of colitogenic mice during intestinal inflammation. In particular, the reduction of predominant butyrate producers including *F. prausnitzii, Roseburia* spp., and *E. hallii* in colitogenic mice correlated with the decreased levels of butyrate and the disease activity. Finally, we showed that exogenous supply of butyrate attenuated intestinal inflammation in murine colitis model, suggesting its protective effect against colitis.

SCFAs are organic fatty acids derived from bacterial fermentation of carbohydrates, dietary fibers, and proteins in the colon. Fermentation process provides energy for microbial growth as well as other metabolites for use by the host. The metabolism of colonic SCFAs was mainly dependent on the availability of fermentable diet and the composition of gut microbiota. In healthy subjects, the production of acetate: propionate: butyrate is approximately in a molar ratio of 60: 20: 20, respectively [25], and such ratio is relatively stable along the colon in the conditions of gut homeostasis. Our SCFAs analysis showed that the average molar ratio of main SCFAs (3.3: 1: 1), and caecal butyrate levels (range: 10-40 mM) in WT mice was very similar to healthy individuals (ratio 3: 1: 1; butyrate range: 11-25 mM) [26], indicating that regulation of butyrate metabolism is fairly constant. However, the levels of colonic SCFAs cannot represent the *in situ* production of total SCFAs, since more than 95% of the SCFAs, especially butyrate, are rapidly absorbed and metabolized by the host [27]. Moreover, the estimated amount of total SCFAs decreased from proximal colon (range: 70-140 mM) to the distal colon (range: 20-70 mM) [28].This suggests the levels of SCFAs likely also reflect the relative absorption capability by colonic enterocytes Consequently, the considerable

imbalance and reduction of colonic SCFAs is indeed a diagnostic parameter to monitor chronic inflammation caused by microbial dysbiosis and impaired colonic epithelium.

Butyrate is produced mainly via butyryl-CoA: acetate CoA transferase enzyme, or less frequently by butyrate kinase [29]. Bacteria that utilize butyryl-CoA: acetate CoA transferase include several Clostridial cluster IV and XIVa species, such as *F. prausnitzii*, *Roseburia* spp., *Eubacterium* and *Butyricicoccus* spp. which generally convert acetate to butyrate. *E. hallii* can also use lactate to produce butyrate, thus have an important role in stabilizing the intestinal microenvironment by preventing the accumulation of lactate [14, 30]. Several studies have reproducibly documented a decrease of butyrate producers, especially *F. prausnitzii*, and more recently *Roseburia hominis* in the gut of IBD patients [5], suggesting a positive probiotic effect of such bacteria for treatment of IBD. In addition, a recent study showed that *Butyricicoccus* bacteria, which exerts anti-inflammatory properties, were significantly lower in the gut of IBD patients than healthy subjects. Administration of *Butyricicoccus* bacteria also attenuated experimental colitis in rat by decreasing production of inflammatory cytokines [31]. Our data suggested that lower abundance of *E. hallii* and *F. prausnitzii* correlates with the disease activity. Accordingly, their presence in ST mice may account for the residual butyrate production detected in these mice during inflammation. Overall, butyrate-producing bacteria present in the colon are phylogenetically diverse. Therefore, further isolation and culture of targeted bacteria will be necessary to elucidate the function of butyrate producers and their interplay between diet and other microbiota.

The critical roles of butyrate on maintaining colonic health prompted researchers to examine their therapeutic effects against intestinal inflammation. SCFA enemas, especially butyrate, are mostly applied and demonstrated a marked improvement in treating bowel disease [10-11]. However, some studies also revealed inconsistent clinical outcomes [12, 32], possibly due to the variation of dosage, frequency and duration of treatments. Moreover, a low compliance rate and discontinuous applications also limited the efficacy of SCFA enema. Therefore, the use of encapsulated organic acids and oral ingestion of fermentable dietary fibers have been explored. Lipid-encapsulated butyrate shows a promise of treating bowel

disease by slowly and continuously release the active components along the whole gastrointestinal tract or targeted release the components in the specific region [33]. Fermentable fibers such as germinated barley [34], inulin [35], and oat bran [36] have also been investigated in several clinical studies and shown to increase faecal SCFAs levels and prolonged remission in IBD patients [37]. These studies help to provide the dietary recommendations for supporting colonic health and disease prevention. Further longitudinal analysis of microbiota composition and functional analysis of the microbial metabolism in a large cohort of IBD patients will help to develop the treatment for IBD.

Materials and Methods

Real-time PCR analysis of microbial 16S rDNA

The abundance of bacterial family and genera in fecal samples was determined by real-time PCR using the EvaGreen qPCR Master Mix (Biotium). Primer pairs specific for 16S rDNA of *Bacteroidaceae* (Bac32F, Bac303R)*, Lachnospiraceae* (Erec482F, Erec870R) *Lactobacillaceae* (Lacto-05, Lacto-04), *Enterobacteriaceae* (Eco1457F, Eco1652R), *Clostridia* Cluster IV (Clep866mF, Clept1240mR), *Ruminococcaceae* (Rflbr730F, Clep866mR), and total bacteria (Eub338F, Eub518R) were described previously [38-39]. Cycling conditions were 40 cycles at 95°C for 10 s, annealing for 10 s and 72°C for 25 s after an initial denaturation at 95°C for 3 min. The annealing temperature for each primer pair is listed in table 1. Quantification values were calculated by the $2^{-\triangle Ct}$ method relative to total bacterial 16S rDNA amplicons. To determine the relative abundance of butyrate-producing bacteria, primers targeting *Faecalibacterium prausnitzii* (FPR-2F, Fprau645R)[3], *Roseburia spp* (RrecF, RrecR) and *Eubacterium hallii* (EhalF, EhalR)[40] were used and quantified by the $2^{-\triangle Ct}$ method relative to total bacterial 16S rDNA [41].

Mouse models and DSS-induced colitis

Conventionally reared C57BL/6 wildtype and sialyltransferase *St3gal4* knockout mice were derived from the same breed and maintained in light-cycled and climate-controlled facility.

All experiments were performed in compliance with the Swiss Animal Protection Ordinance and approved by the Veterinary Office of the Canton of Zurich, Switzerland. For DSS-induced colitis, 6-7 week-old, gender-matched mice were treated with 3- 3.5% (w/v) of DSS (MW=36-50 kDa; MP Biomedicals) in drinking water for 5 days, followed by a supply of normal water until sacrifice of the animals. Body weight and physical activity were monitored daily. For antibiotic treatment, 3-week-old mice were provided with sterile drinking water supplemented with vancomycin (0.5 g L^{-1}), streptomycin (1 g L^{-1}), neomycin (1 g L^{-1}), or penicillin (0.5 g L^{-1}) plus aspartame (0.25%, w/v) for 3 weeks before DSS treatment.

Heteroduplex DNA amplification

Mouse fecal DNA was isolated from stool samples using the QIAamp DNA Stool Mini Kit (Qiagen) according to manufacturer's instructions. Lysis temperature was increased to 95°C for 5 min to ensure complete cell lysis of Gram-positive bacteria. Extracted DNA were subjected to PCR using the 16S rDNA universal heteroduplex analysis (HDA) primers HDA1-GC and HDA2 [42], U968-GC and L1401r [43], Bac32GC-f, and Bac303-r [44]. GC clamp was attached at the 5' end of wither forward or reverse primer. Additional primers used for specific bacterial group amplification are listed in table 1. PCR conditions were 5 min of initial denaturation, followed by 30 cycles of 30 s at 95 ^{o}C, 30 s at 56 ^{o}C and 1 min at 72 ^{o}C, and the final elongation at 72 ^{o}C for 5 min. PCR products were verified by agarose gel electrophoresis before being subjected to DGGE analysis.

Denaturing gradient gel electrophoresis (DGGE)

The amplified 16S rDNA products were subjected to DGGEusing an INGENYphorU (Ingeny International BV) system as previously described [45]. In brief, the system was equipped with a 4.5% of polyacrylamide gel with denaturant in the range of 40-60%, in which 100% denaturant is equivalent to 7M of urea and 40% of formamide. Electrophoresis was carried out at 130 V for 7 h at 58 ^{o}C. Polyacrylamide gel was stained with GelRed nucleic acid stain (Biotium) for 30 min, destained in distilled water and viewed under UV light. DNA bands of

interest were excised, and resuspended in ultrapure water at 4 ^{o}C overnight. The extracted DNA was reamplified using the same primer and PCR conditions. To purify the bacterial DNA, products were reloaded on a denaturant gradient and separated before being subjected to reamplification using the same PCR conditions. The DNA product was purified using NucleoSpin gel purification kit (Macherey-Nagel, Germany) before sequencing (Microsynth, Switzerland). Bacterial taxonomy was assigned using the Ribosomal database Project (RDP) classifier [46].

Quantitative analysis of caecal SCFA

Mouse caecal contents were isolated and centrifuge at 4°C and 15,000 x g for 10 min. The supernatant was diluted with ultrapure water and filtered through a 0.45 µm nylon filter (Infochroma AG), and stored in -80°C before analysis. SCFA analysis was performed by HPLC using cation-H refill cartridge (30 x 4.6 mm) and Aminex® HPX-87H column at a flow rate of 0.4 ml min^{-1} for 60 min and eluted with 10 mM sulphuric acid solution [47] . Quantification was done by refractive index detection. Each sample runs in duplicate, and the concentration (mM) was calculated by integral area comparison with authentic standard solutions.

Butyrate supplementation in mice

For oral supplementation, 7-week old WT and ST male mice received a SCFA mixture in drinking water containing 100 mM sodium butyrate (Sigma-aldrich) plus 0.25% sweetener for 7 days together with 2.5-3 % DSS for the first 5 days. Control mice received normal drinking water and DSS. For rectal administration, 7-week old WT male mice were administered sterile sodium butyrate solution and challenged with 3% DSS for 5 days. Mice were mildly anesthetized by isoflurane inhalation and received rectal instillation of 100 µl of sterile PBS or 100 mM sodium butyrate by polyethylene catheter PE-50 (Becton Dickson, New Jersey). Both solutions were adjusted to pH 6.5. Mice were kept in a vertical position head-down for 2 min to avoid excretion of the enema solution. After rectal administration, body weight and sign of the dehydration were monitored for the duration of the experiment.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5.0 software (San Diego, CA). The results were tested by two-tailed unpaired Student's t-test and ANOVA Bonferroni multiple comparison test. Difference reaching P values below 0.05 were considered significant. Results are presented as mean ± SEM unless specified.

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Author contributions

T.H. and Y.L.H. designed the study; Y.L.H. performed the experiments; A.D. performed HPLC analysis of SCFA; T.H., Y.L.H., and C.C. analyzed data; All authors discussed the results and Y.L.H. and T.H. wrote the manuscript.

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Table 1. Primer list of quantitative microbial analysis and DGGE profiling

Primers for quantitative microbial analysis

Bacteria	Primers	Sequences 5'-3'	Size (bp)	Anneal (^{o}C)
Bacteroidetes	Bac32F Bac303R	AACGCTAGCTACAGGCTT CCAATGTGGGGGACCTTC	276	55
Clostridia Cluster IV	Clep866F Clept1240R	TTAACACAATAAGTWATCCACCTGG ACCTTCCTCCGTTTTGTCAAC	314	64
Enterobacteriaceae	Eco1457F Eco1652R	CATTGACGTTACCCGCAGAAGAAGC CTCTACGAGACTCAAGCTTGC	195	62
Lactobacillaceae	F_Lacto 05 Lacto 04	AGCAGTAGGGAATCTTCC CGCCACTGGTGTTCYTCCATATA	383	66
Lachnospiraceae	Erec482F Erec870R	CGGTACCTGACTAAGAAGC AGTTTYATTCTTGCGAACG	429	55
Ruminococcaceae	Rflbr730F Clep866mR	GGCGGCYTRCTGGGCTTT CCAGGTGGATWACTTATTGTGTTAA	157	60
F. prausinitzii	FPR-2F Fprau645R	GGAGGAAGAAGGTCTTCGG AATTCCGCCTACCTCTGCACT	248	58
Roseburia spp	RrecF RrecR	GCGGTRCGGCAAGTCTGA CCTCCGACACTCTAGTMCGAC	81	60
Eubacterium hallii	EhalF EhalR	GCGTAGGTGGCAGTGCAA GCACCGRAGCCTATACGG	276	60
Primers for DGGE profiling				
Universal 16S (V3)	HDA1-GC HDA2	GC-clamp + ATCCCTACGGGAGGCAGCAGT GTATTACCGCGGCTGCTGGCA	200	56
Universal 16S (V6-V8)	U968-GC L1401r	GC-clamp + ACGCGAAGAACCTTAC GCGTGTGTACAAGACCC	430	56
Clostridia cluster IV	Clep-F GC-Clep-R3	GCACAAGCAGTGGAGT GC-clamp + CTTCCTCCGTTTTGTCAA	239	55
Bacteroida- ceae	Bac32-GC Bac303r	GC-clamp + AACGCTAGCTACAGGCTT CCAATGTGGGGGACCTTC	276	53

GC clamp: CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGC ACGGGGGG

Table 2. Levels of SCFAs in caecal samples of WT and ST mice

Conc (mM)	WT					ST	
Treatment	Ctrl	Stp	Van	Pen	DSS	Ctrl	DSS
Acetate	80.2 ± 4.4 [a]	23.1 ± 3.5 [b]	12.8 ± 5.4 [b]	7.9 ± 0.7 [b]	25.7 ± 2.1 [b]	61.6 ± 3.0 [a]	48.3 ± 3.6 [b]
Propionate	24.7 ± 1.9 [a]	5.5 ± 0.8 [b]	1.2 ± 1.0 [c]	0.2 ± 0.2 [c]	2.9 ± 1.1 [c]	24.3 ± 2.0 [a]	11.0 ± 1.9 [b]
Butyrate	24.1 ± 2.6 [a]	7.7 ± 1.0 [b]	0.2 ± 0.2 [c]	0.2 ± 0.2 [c]	0.4 ± 0.3 [c]	9.3 ± 0.8 [a]	8.4 ± 1.0 [a]
Lactate	10.8 ± 0.4 [a]	3.0 ± 0.3 [b]	9.5 ± 1.6 [a]	8.5 ± 1.3 [a]	14.9 ± 1.6 [a]	9.1 ± 0.9 [a]	11.2 ± 2.5 [a]

SCFAs, short-chain fatty acids. The data is represented as the mean ± SEM from two independent experiments, N=6-8. Different letters in the same row indicate which treatments are statistically significant ($p < 0.05$, ANOVA multiple Bonferroni multiple comparison test)

Figure legends

Figure 1. Antibiotic effects on DSS-induced colitis. 7-week-old gender-matched WT and ST mice were challenged with DSS (3.3%, w/v) in drinking water for 5 days, followed by a supply of normal drinking water until sacrifice of the animals. For antibiotic treatment, mice were treated with **(a)** 1 g L^{-1} neomycin (Neo), **(b)** 0.5 g L^{-1} vancomycin (Van), **(c)** 0.5 g L^{-1} penicillin (Pen) or **(d)** 1 g L^{-1} streptomycin (Stp) for 3 weeks before DSS challenge. Control mice received normal drinking water and DSS. Body weight and physical activity were monitored daily. **(e)** Colon length was measured at day 7 after initiation of DSS challenge and given as percentage to the colon length of mice without any treatment. The data is represented as mean ± SEM, N=6-8. The letters above the bars indicate which treatments are statistically significant ($p < 0.05$, ANOVA Bonferroni multiple comparison test).

Figure 2. Antibiotic effects on the intestinal microbiota. The compositions of the intestinal microbiota of control and antibiotic-treated mice were determined by real-time PCR targeting specific 16S rDNA bacterial phylogenetic groups. The relative abundance of **(a)** *Clostridia* cluster IV **(b)** *Clostridia* cluster XIVa *(Lachnospiraceae*) **(c)** *Ruminococcaceae* **(d)** *Enterobacteriaceae* **(e)** *Bacteroidaceae,* and **(f)** *Lactobacillaceae* was quantified using $2^{-\triangle Ct}$ method to the total bacteria 16S rDNA. The data is represented as median from two

independent experiments, N=5-7. Statistical difference versus control group *p <0.05, (ANOVA Bonferroni multiple comparison test).

Figure 3. Quantitative analysis of caecal SCFAs. At day 7 after initiation of DSS treatment, mice caecal fluid was collected. The levels of SCFAs in caecal fluid were analyzed by HPLC. **(a)** The concentrations of acetate, propionate, butyrate and lactate were calculated by comparing to the authentic standard solution. Each sample runs in duplicate and the data is represented as mean ± SEM from three independent experiments, N=15. **(b)** Caecal fluid samples from control and antibiotic-treated WT mice were collected. The levels of SCFAs and lactate were analyzed by HPLC. Dta is represented as mean ± SEM, N=6-8. The letters above the bars indicate which treatments are statistically significant (p <0.05, ANOVA multiple Bonferroni multiple comparison test). **(c)** The molar ratio of caecal SCFAs in WT and ST mice, and **(d)** antibiotic-treated WT mice was given as percentage of total SCFAs obtained from a pool of 6 mice per group.

Figure 4. Quantitative analysis of butyrate-producing bacteria. Relative abundance of butyrate-producing bacteria in mice was determined by real-time PCR using specific 16S rDNA primers targeting **(a)** Clostridial XIVa **(b)** *F. prausnitzii* **(c)** *Roseburia spp* and **(d)** *Eubacterium hallii,* and quantified by the $2^{-\triangle Ct}$ method. A group of 6-7 week-old WT and ST mice were treated with or without DSS for 5 days. For antibiotic treatment, WT mice were treated streptomycin (Stp 1 g L^{-1}) or vancomycin (Van 0.5 g L^{-1}) for 3 weeks. The data is represented as median values. Each point indicates an individual mouse from two independent experiments, N=6-7, statistically significant versus WT control group, *p value < 0.05 (ANOVA multiple Bonferroni multiple comparison test).

Figure 5. The effects of butyrate administration to mice in DSS-induced colitis. (a) WT mice were treated with 100 mM of butyrate in drinking water for 7 days, and challenged with 2.5% DSS for the initial 5 days. Body weight was monitored daily. **(b)** ST mice were treated with 100 mM of butyrate in drinking water for 7 days, and challenged with 3% DSS for the initial 5 days. Control mice received drinking water and DSS. The data is represented as mean ± SEM,

N=5. **(c)** WT mice were rectally administered with 100 mM sterile sodium butyrate or PBS at time points marked with black arrows and challenged by 3% of DSS in drinking water. **(d)** colon length was measured at day 7 after initiation of DSS challenge. The data is represented as mean ± SEM, N= 4-6. ns, not significant (two-tailed Student's t test).

Figure 1

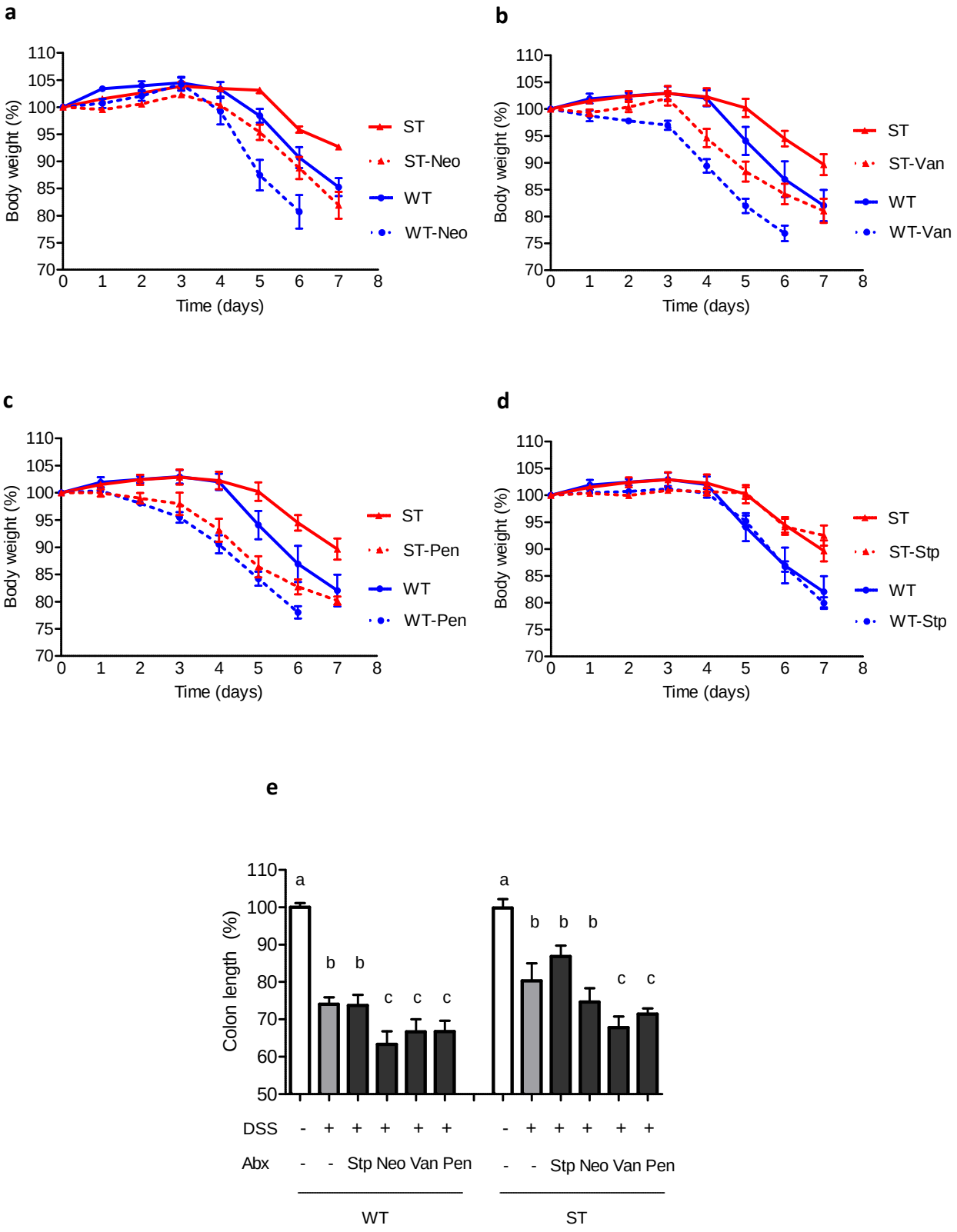

a
b
c
d
e
Body weight (%)
Time (days)
ST
ST-Neo
WT
WT-Neo
ST-Van
WT-Van
ST-Pen
WT-Pen
ST-Stp
WT-Stp
Colon length (%)
DSS
Abx
Stp Neo Van Pen
WT
ST

Figure 2

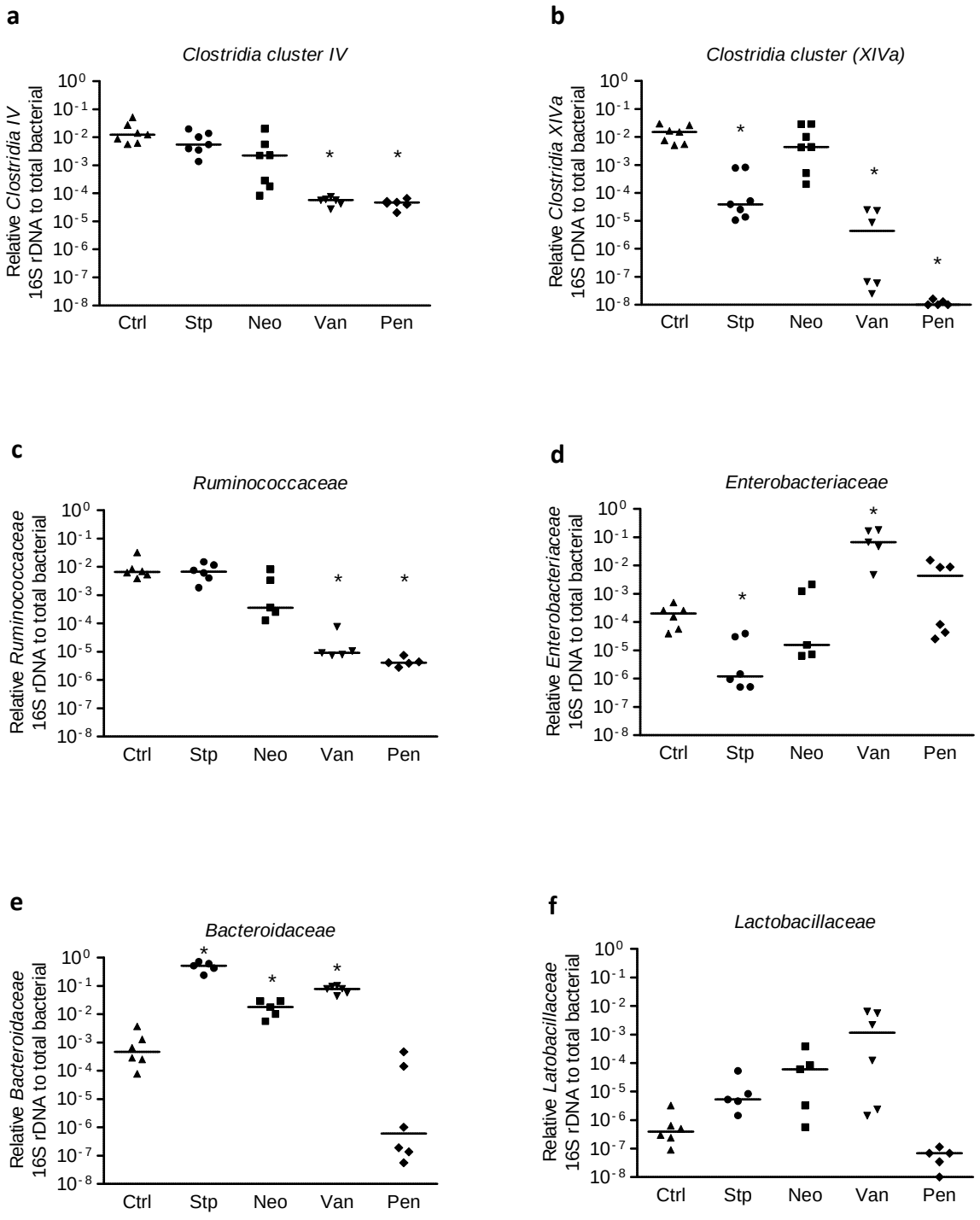

a
Clostridia cluster IV
Relative Clostridia IV 16S rDNA to total bacterial
b
Clostridia cluster (XIVa)
Relative Clostridia XIVa 16S rDNA to total bacterial
c
Ruminococcaceae
Relative Ruminococcaceae 16S rDNA to total bacterial
d
Enterobacteriaceae
Relative Enterobacteriaceae 16S rDNA to total bacterial
e
Bacteroidaceae
Relative Bacteroidaceae 16S rDNA to total bacterial
f
Lactobacillaceae
Relative Latobacillaceae 16S rDNA to total bacterial
Ctrl
Stp
Neo
Van
Pen

Figure 3

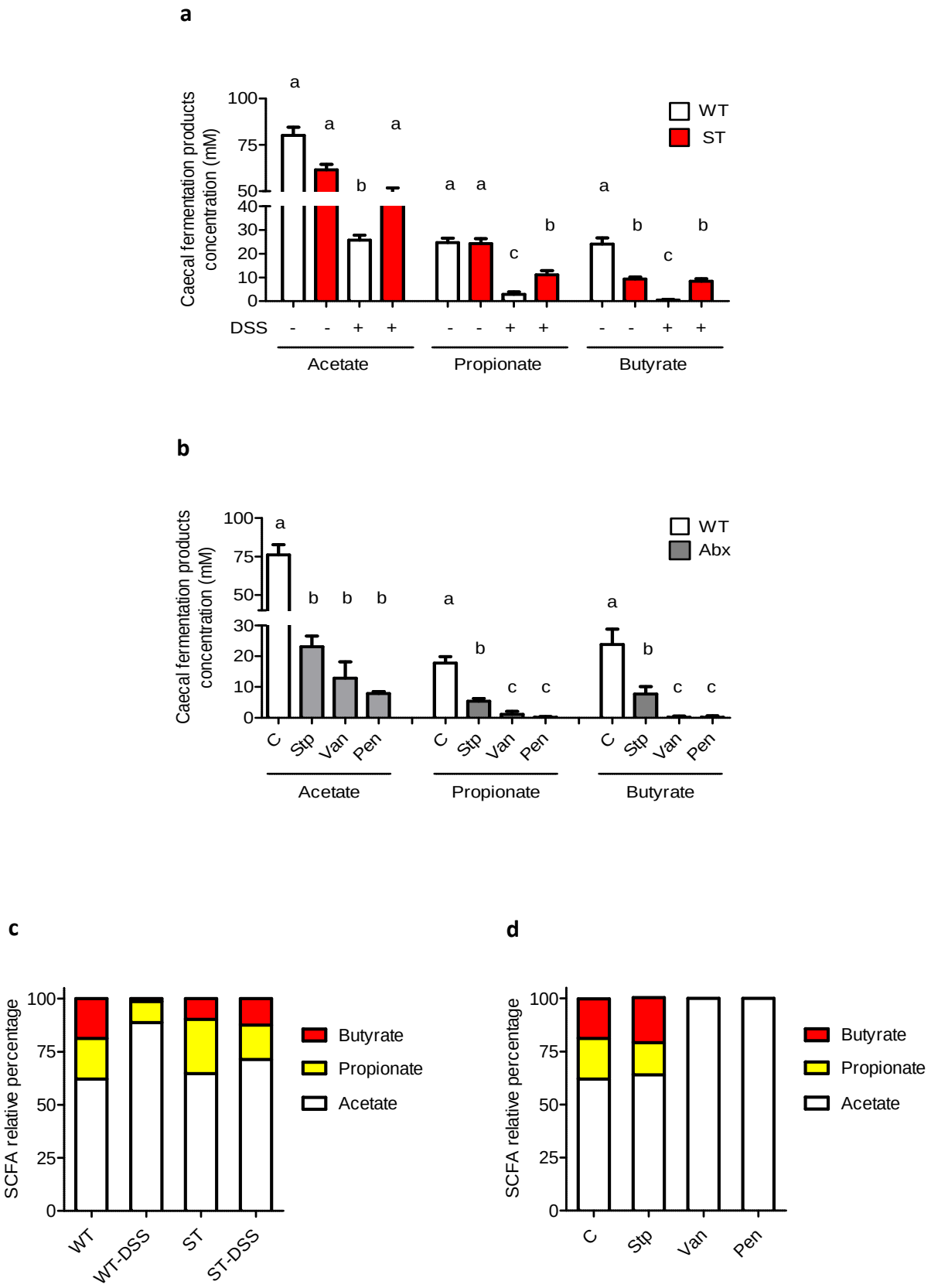

a
Caecal fermentation products concentration (mM)
WT
ST
DSS
Acetate
Propionate
Butyrate
b
Caecal fermentation products concentration (mM)
WT
Abx
C
Stp
Van
Pen
Acetate
Propionate
Butyrate
c
SCFA relative percentage
Butyrate
Propionate
Acetate
WT
WT-DSS
ST
ST-DSS
d
SCFA relative percentage
Butyrate
Propionate
Acetate
C
Stp
Van
Pen

Figure 4

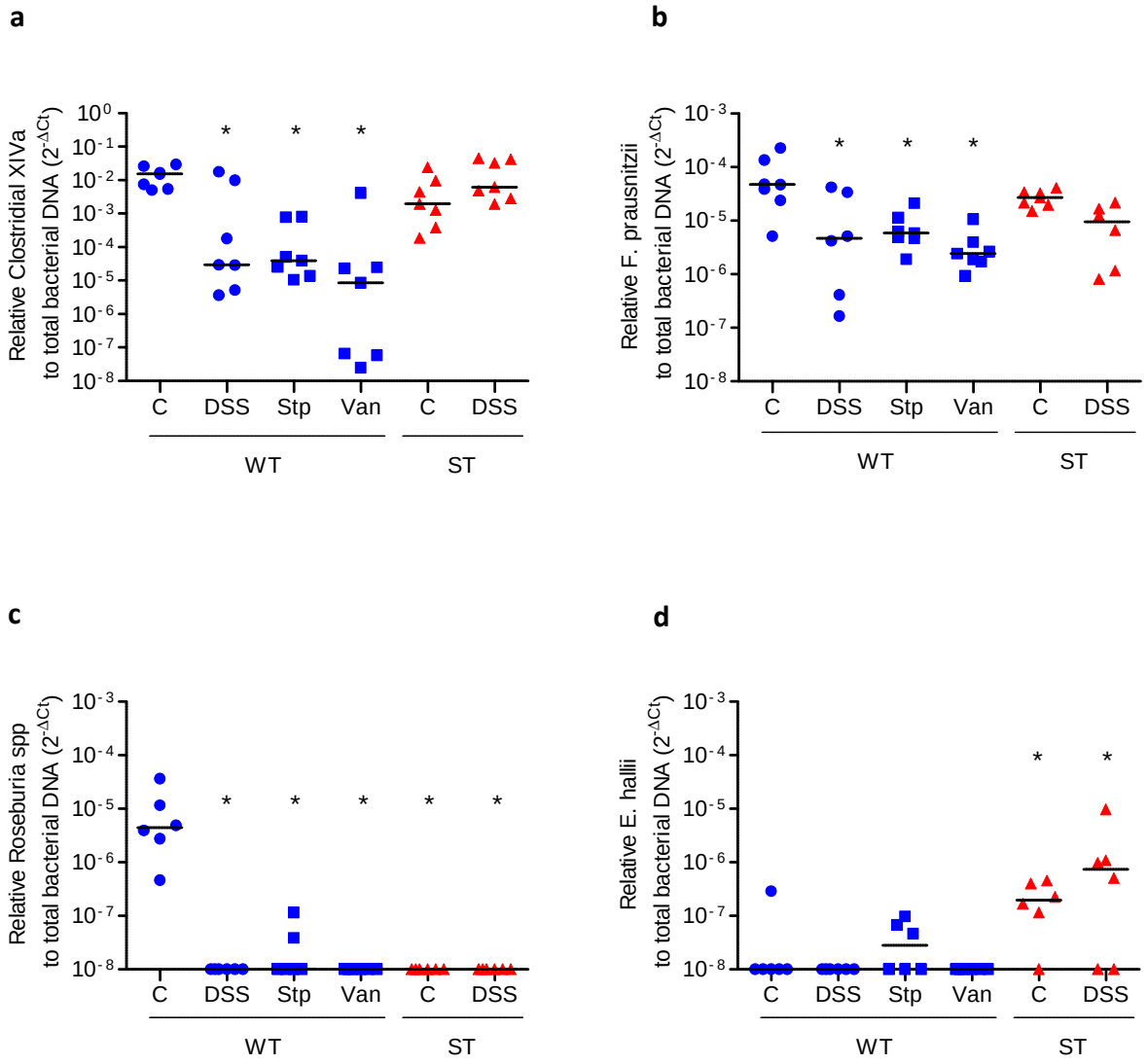
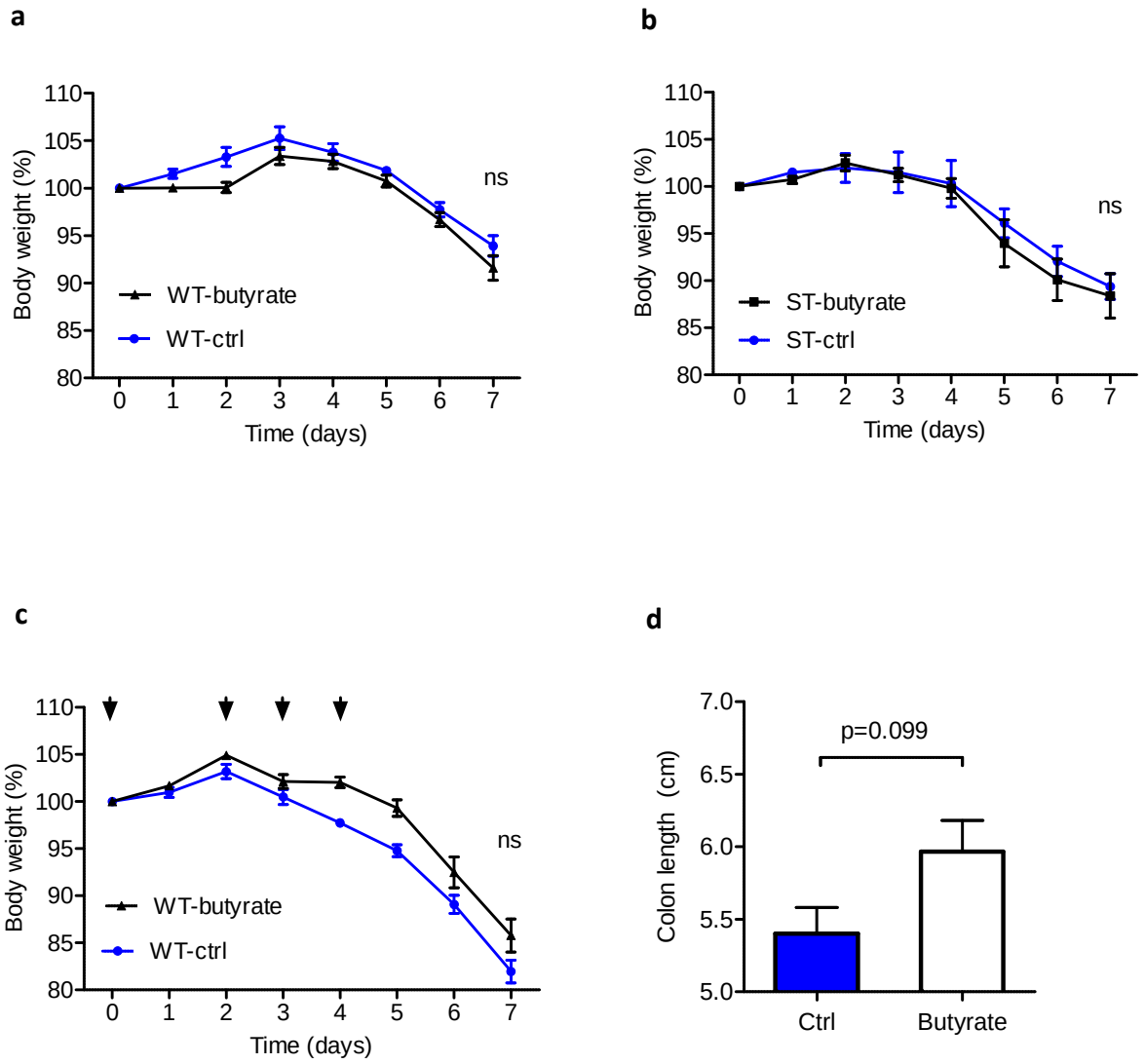
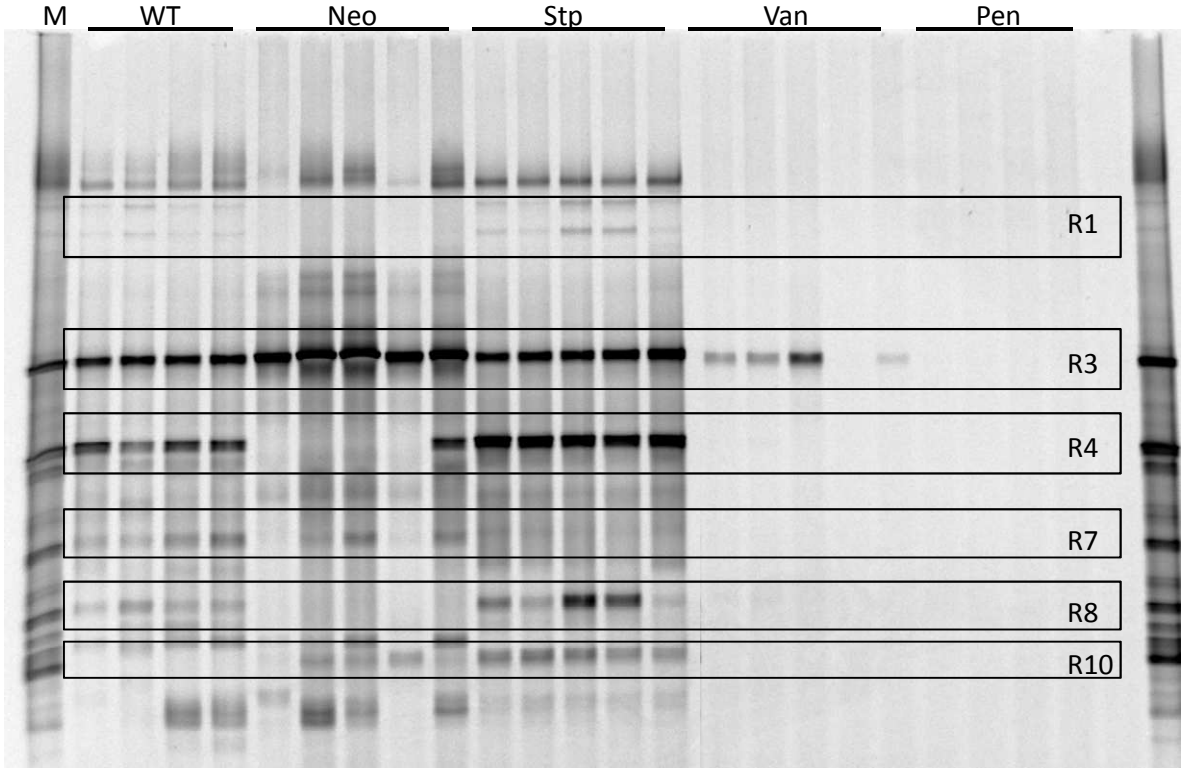

a
Relative Clostridial XIVa to total bacterial DNA ($2^{-\Delta Ct}$)
10^{0} 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7} 10^{-8}
C DSS Stp Van C DSS
WT ST
b
Relative F. prausnitzii to total bacterial DNA ($2^{-\Delta Ct}$)
10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7} 10^{-8}
C DSS Stp Van C DSS
WT ST
c
Relative Roseburia spp to total bacterial DNA ($2^{-\Delta Ct}$)
10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7} 10^{-8}
C DSS Stp Van C DSS
WT ST
d
Relative E. hallii to total bacterial DNA ($2^{-\Delta Ct}$)
10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7} 10^{-8}
C DSS Stp Van C DSS
WT ST

Figure 5

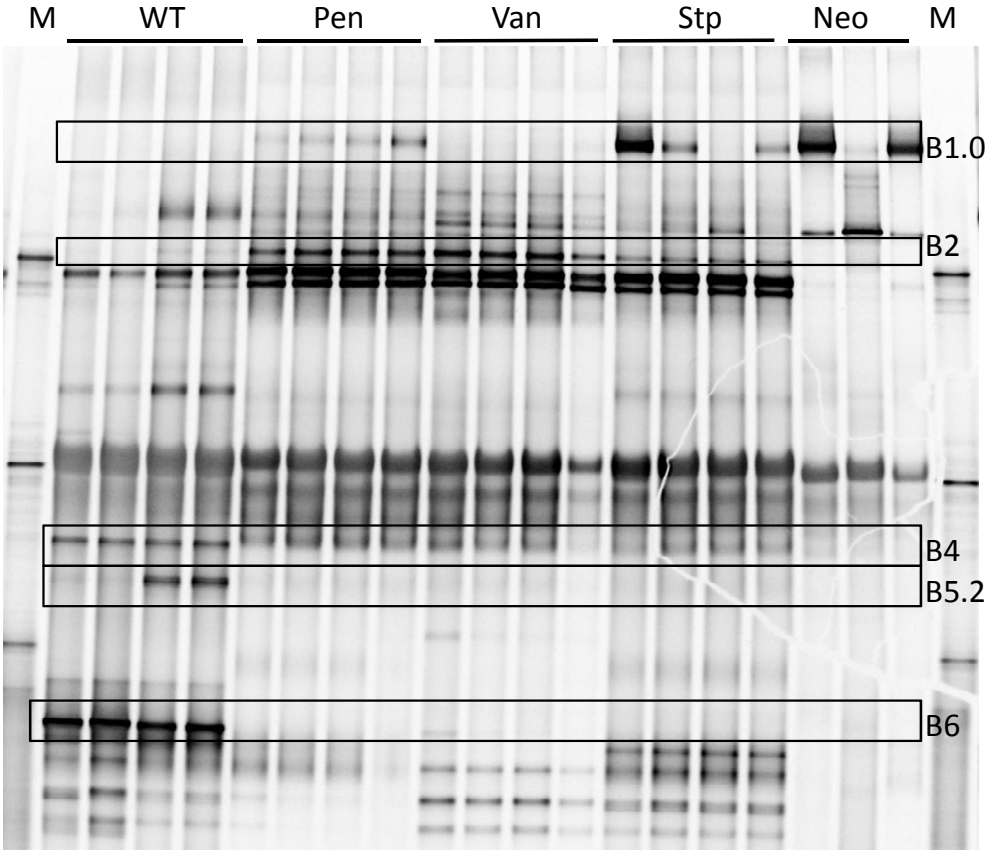

a
Body weight (%)
110
105
100
95
90
85
80
0 1 2 3 4 5 6 7
Time (days)
ns
WT-butyrate
WT-ctrl
b
Body weight (%)
Time (days)
ns
ST-butyrate
ST-ctrl
c
Body weight (%)
Time (days)
ns
WT-butyrate
WT-ctrl
d
Colon length (cm)
7.0
6.5
6.0
5.5
5.0
p=0.099
Ctrl
Butyrate

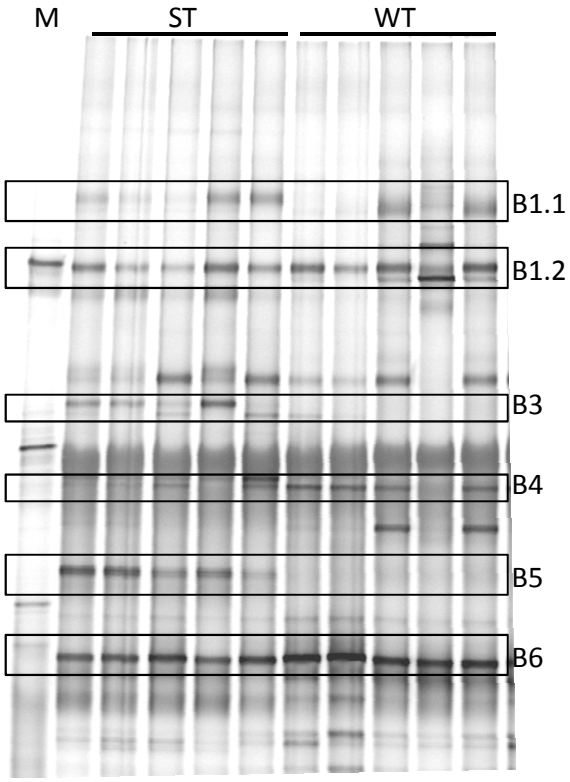
Supplementary Figure 1



Supplementary Figure 1. Effects of antibiotic to the diversity of gut microbiota. DGGE profiles of bacterial 16S rDNA amplified from fecal DNA of antibiotic-treated WT mice (upper panel) and ST mice (lower panel). The bacterial 16S rDNA was amplified by specific primer targeting for the *Clostridia* cluster IV group. Each lane represents DNA of fecal bacteria from individual mouse. Horizontal box mark the position of a corresponding member of *Ruminococcaceae* spp (R1: *R. bromii*, R3: *R. bromii*, R4: *Oscillibacter* spp., R7: *R. callidus*, R8: *Oscillibacter* spp., R10: *R. champanellensis*, M: marker)

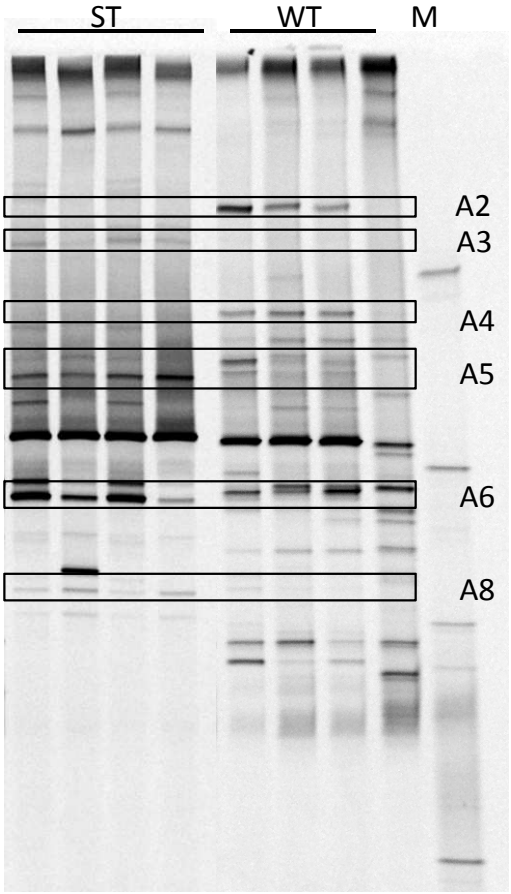
Supplementary Figure 2





Supplementary Figure 2. Effects of antibiotic to the diversity of gut microbiota. DGGE profiles of bacterial 16S rDNA amplified from fecal DNA of antibiotic-treated WT mice (upper panel) and ST mice (lower panel). The bacterial 16S rDNA was amplified by specific primer targeting for the *Bacteroidetes* group. Each lane represents DNA of fecal bacteria from individual mouse. Horizontal box mark the position of a corresponding member of *Bacteroidaceae* spp. (B1.0-B1.2: *B. acidifaciens*, B2: *B. vulgatus*, B3: *B. uniformis*, B4, *Porphyromonadaceae* spp., B5: *Parabacteroides distasonis*, B6: *Prevotellaceae s*pp. M: marker)

Supplementary Figure 3



Supplementary Figure 3. Effects of antibiotic to the diversity of gut microbiota. DGGE profiles of bacterial 16S rDNA amplified from fecal DNA of WT mice and ST mice. The bacterial 16S rDNA was amplified by universal primer (U968GC and L1401r). Each lane represents DNA of fecal bacteria from individual mouse. Horizontal box mark the position of a corresponding bacterium. (A2: Segmented filamentous bacteria, A3: *Ruminococcus gauvreauii*, A4: *Clostridium* spp., A5: *Clostridium* spp., A6: *Ruminococcus* spp. A8: Butyrate-producing bacteria, M: marker)

GENERAL DISCUSSION

Several recent studies have reported that intestinal inflammatory diseases are frequently accompanied by microbial dysbiosis and impaired mucosal functions. However, the mechanisms underlying such changes remain largely unknown. Dietary carbohydrates are a major source of nutrients that profoundly affect the gut microbiota. The impact of complex carbohydrates on microbial composition is based on the production of specific hydrolases, which enable some bacterial species to utilize the breakdown products as nutrients, thereby conferring a growth advantage over bacteria that cannot process complex glycans. We showed that sialic acids provided by milk oligosaccharides and released from host mucosal tissues confers a proliferative advantage for *E. coli* during lactation and intestinal inflammation. Moreover, we identified an increased availability of intestinal sialic acids during inflammation was mediated by *Bacteroides vulgatus*-derived sialidase, which enables the sialic acid-dependent proliferation of *E. coli* during inflammation. By application of an *nanT* mutant *E. coli* strain and the inhibition of sialidase, we confirmed the essential contribution of sialic acid catabolism on promoting microbial dysbiosis as manifested by *E. coli* expansion.

The role of glycosylation in mucosal immunity

Mucosal surface is covered with a thick layer of mucus that is decorated by a considerable amount of glycans. Decreased glycosylation on mucins has been documented in patients with IBD, suggesting an increased mucosal exposure to bacteria and may potentially trigger inflammation [1]. Given the richness of sialylation on intestinal mucins, a variety of bacteria and viruses has targeted the host-derived sialylated glycans for adhesion, degradation and infection. Many commensal and pathogenic bacteria have been shown to secret sialidases which release free sialic acids from the mucosa[2]. The extracellular sialidases not only enhance bacterial survival through nutritional benefit but also increase bacterial adhesion by unmasking the underlying cryptic ligands [3]. Moreover, the sequential degradation of mucosal glycans by bacterial glycosidases requires the initial removal of sialic acids before further digestion. This explains an increasing sialic acid gradient from ileum to colon as a

mechanism to separate the underlying mucosal epithelium from a tremendous amount of mucin-degrading bacteria and extracellular glycosidases in the colon [4]. Structural analysis on mucosal sialoglycans reveals other sialic acid derivatives, such as mono- and di-O-acetylated sialic acids, which are also prevalent in human. The O-acetylation of sialic acids has been shown to significantly inhibit the rate of sialic acids cleavage by bacterial sialidases, therefore reinforces the protection from bacterial degradation. However, intestinal bacteria respond by producing sialate O-acetylesterase that removes the O-acetylation (carboxylic ester group) of sialic acids [5]. The subsequent removal of O-acetylation on sialic acids facilitates the degradation of sialoglycans by sialidases, suggesting the role of sialate O-acetylesterase on regulating sialic acid metabolism.

Our proteomic analysis of microbial glycosidases also identified a bacterial-derived sialate O-acetylesterase in the caecal fluid of colitogenic mice. This finding was consistent to a previous study showing that an increased sialate O-acetylesterase activity was observed in the fecal extract of UC patients [6], and later confirmed the enzyme activity in *E. coli* K1 strain [7]. Interestingly, by fluorometric-HPLC analysis of caecal sialic acids and their derivatives from mice, we identified high levels of O-acetylated sialic acid (Neu5,9Ac2) and low levels of Neu5Gc. However, whether these sialic acid derivatives also confer a proliferative advantage to *E. coli* during inflammation, and whether the release of sialic acid derivatives requires different sialidases remains to be investigated. Collectively, bacterial-producing sialidases and sialate O-acetylesterase both show profound effects on mediating sialic acids catabolism and hence regulate the sialic acid-dependent proliferation of bacteria.

Recent meta-analysis of genome-wide association studies have identified more than hundreds of genetic susceptible loci that are linked to IBD pathogenesis [8-9]. These studies highlight the importance of epithelial barrier functions, innate receptors, ER stress, and autophagy in the pathogenesis of IBD. Of particular interest, the loss-of-function alleles of the α1,2-fucosyltransferase *FUT2* gene and single nucleotide polymorphisms are strongly associated with CD pathogenesis [10]. Fucosyltransferase 2, which regulates mucosal expression of ABO blood group antigens, has been shown to maintain the integrity of

epithelial barrier, thereby influenced host-microbiota interaction [11]. Studies on *FUT2* deficient mice also showed an increased susceptibility to the development of chronic inflammation [12], and studies on *FUT1* gene polymorphisms showed an increased susceptibility to fimbriated *E. coli* adhesion and infection [13]. Moreover, innate immunity is also involved in the regulation of mucosal fucosylation. A recent study has described systemic exposure to TLR-ligands leads to a rapid α1,2-fucosylation of intestinal epithelial cells driven by IL-22. The nature of intestinal fucosylation seems to be a protective mechanism to maintain the balance between host immune response and microbiota during bacterial infection [14]. Of note, our study also identified a robust fucosidase activity in the caecal fluid of mice, therefore bacterial-derived fucosidase is also likely to play a potential role in digestion of mucosal glycans and mediating the intestinal inflammation. Accordingly, it is interesting to address whether intestinal sialylation also responds similarly to the bacterial and environmental stimuli. Besides fucosylation and sialylation on mucosal glycans, the importance of mucin O-glycosylation has also been demonstrated in several glycosyltransferases-deficient mice models, in which impaired mucin O-glycosylations on core 1, 2, and 3 structures increased the susceptibility to colitis in mice [15-17].

For the treatment of microbial dysbiosis and intestinal inflammation, we showed that daily administration of the sialidase inhibitor, Neu5Ac2en, effectively inhibits the intestinal sialidase activity and reduces *E. coli* expansion in mice, whereas other neuraminidase inhibitors, zanamivir and oseltamivir showed no inhibition to bacterial sialidases. Therefore, it will be interesting to examine and compare the inhibitory potential of other sialidase inhibitor analogues, such as 4-substituted Neu5Ac2en derivatives, to a variety of Bacteroides-derived sialidases. The potent sialidase inhibitor in reducing the outgrowth of *E. coli* during inflammation may also be used as a potential treatment against other diseases triggered by pathogenic *E. coli* in gastrointestinal or urinal tract infection.

Overall, the advancement of glycomics is now making it more feasible to study the complex glycome in response to environmental or microbial stimuli. The longitudinal analysis of mucosal glycome and microbiome helps us to better understand host-microbiota interaction in a variety of intestinal inflammatory diseases. In particular, it might be of a great promise to

develop a novel therapeutic by regulating glycan metabolic pathway.

The role of gut microbiota in mucosal immunity

The impact of the gut microbiota on mucosal immunity is widely studied in the context of IBD. Accumulating evidences suggest the essential contribution of gut microbiota in driving inflammation in IBD. However, characterization of the specific microbial signature with disease is greatly influenced by inter-individual variability, such as genetic background and individual's gut microbiome [18]. The studies based on discordant disease status of twin pairs may be a valuable approach to address this question. Microbial alteration in CD patients confirmed the reduction of bacterial diversity and disappearance of core bacteria, including *Faecalibacterium* and *Roseburia*, as well as expansion of Enterobacteriaceae [19]. Notably, both *Faecalibacterium* and *Roseburia* spp. belong to butyrate-producing bacteria, which exhibit potent anti-inflammatory effects in mouse model [20]. By contrast, Enterobacteriaceae, in particularly *E. coli*, are associated with disease progression, reproducibly observed in patients with both UC and CD.

Our microbial analysis based on murine colitis model also identified an increased abundance of *Enterobacteriaceae* in colitogenic mice. Further isolation of dominant bacteria and real-time PCR analysis confirmed the expansion of *E. coli* strain in colitogenic mice. Commensal *E. coli* is the most prominent aerobic bacteria colonized in the vicinity to the colonic mucosa and is believed to establish a colonization barrier against pathogenic invaders. The beneficial effects of *E. coli* have been proven in several randomized controlled clinical studies in which the maintenance of remission by *E. coli* strain Nissle 1917 is equivalent to a standard mesalazine treatment in IBD patients [21-22]. The strain-specific characteristic of *E. coli* Nissle 1917 is a point mutation in the gene for LPS O-antigen synthesis, leading to a repeating O6 subtype antigen structure and a unique immunomodulating effect [23]. Additionally, *E. coli* Nissle 1917 shows strong vitality and fitness by expressing enterobactin for efficient iron uptake and H1 flagella for increasing mobility [24]. The expression of enterobactin has been recently confirmed as a survival advantage for *E. coli* to surpass host immune system during intestinal inflammation [25].

However, the phenotypes of *E. coli* are extremely diverse, some of the *E. coli* strains cause severe infections in humans, such as enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC). Differentiation among these pathogenic *E. coli* requires detection of specific virulence factors. However, by PCR analysis, we did not detect any specific virulence genes including heat-labile enterotoxin (*LT*), heat-stable enterotoxin (*STII*), shiga-toxin (*Stx*), invasion plasmid antigen H (*ipaH*) and intimin (*eae*) in our *E. coli* isolate (EHV2) from colitogenic mice. This might explain why the reconstitution of EHV2 to WT mice did not directly drive the intestinal inflammation. Interestingly, we found that EHV2 harbors a 4.6 kbp plasmid encoding gene homologous to colicin. Colicin is a plasmid-encoded protein toxin produced by, and active against, *E. coli* or related species. Colicin is released into extracellular medium and forms a pore structure in inner membrane thereby exerts cytotoxic effect to other bacteria [26]. EHV2 harbors colicin-encoded plasmid most likely in order to reduce the growth competition against other bacteria. However, whether EHV2 strain also influences on host mucosal immunity needs to be further investigated. Characterization of specific *E. coli* serotype (O, H and K antigens) and whole-genome sequencing allow us to better understand the uniqueness of this isolate strain.

Conclusion and future perspective

Glycosylation is a key post-translational modification of proteins and lipids, and plays essential roles in regulating a variety of physiological pathways. Structural complexity of the glycans hampers the elucidation of the interplay between host glycosylation and the gut microbiota in gastrointestinal physiology. Taking advantage of advancement in glycan synthesis and next-generation sequencing, we demonstrate for the first time that host-derived sialylation confers a critical growth advantage for *E. coli* during intestinal inflammation, and unravel a fundamental mechanism how glycan metabolism shapes the gut microbiota.

However, no evidence gathered to date suggested that a single bacterial strain can directly initiate the onset of inflammatory bowel disease. Therefore, a large-scale and longitudinal

analysis of microbiota composition in IBD patients is required to evaluate the contributions of the gut microbiota on IBD pathogenesis. In addition to bacteria, fungi and viruses also compose the human gut microbiota, thereby should not be neglected in the investigation [27]. Recent studies have demonstrated interactions between enteric fungi and host innate immunity and also identified that a genetic polymorphism of fungal recognition is correlated with the severe form of UC [28]. Additionally, emerging evidence has revealed that the enteric virome is altered in patients with IBD, with disease specific changes observed between UC and CD [29]. The increasing richness and diversity of enteric virome, in particular, a marked expansion of bacteriophages was observed in IBD patients, thereby may affect the diversity of the gut bacteria [30]. Future studies will be focused on elucidating the complex interplay between Fungal, viral, and bacterial community, and their contributions to the host immune system and disease pathogenesis.

Taken together, variations in diet, host genetics, and environmental stimuli create a dynamic and heterogeneous environment for the microbial community in the gut. Attempts to characterize the fluctuations of host glycosylation and the gut microbiota between healthy and disease state can provide us potential opportunities to develop the diagnostic tools and relevant therapies for intestinal inflammatory diseases.

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